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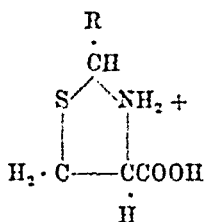
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Errata.

- P. 109, line 1, from above, for "did not accelerate" read "accelerated".
 P. 116, line 7, from above, for "dit not facilitate" read "facilitated".
 P. 116, line 2, from below, the formula to the left read:



INTRODUCTION.

The intimate cultural and personal relations between the Scandinavian countries, Denmark, Finland, Norway and Sweden, already long ago led to the foundation of common scientific journals. In this way the contributions from these countries were to a great extent made easily accessible to the Scandinavian scientists themselves, and collaboration and mutual understanding were facilitated. Later on, such journals were made generally available by being published in English, French or German, thus giving an opportunity to scientists from all parts of the world to become acquainted with the work performed in Scandinavia. There can be no doubt that this has led to an increased knowledge and appreciation of what has been done and stimulated to new efforts.

Within the medical sciences the first journal of the type mentioned was started in 1869 under the title *Nordiskt Medicinskt Arkiv*. It became necessary, however, to divide it into two archives, one for medicine and one for surgery. From 1919 these have appeared as *Acta medica scandinavica* and *Acta chirurgica scandinavica*. A large number of other *Acta* has been added to those already existing, there being at present 11 such journals, covering different fields within the medical sciences.

With regard to physiology and related subjects it was found at an early date that a special journal was highly desirable. Several difficulties made it impossible, however, to start such an archive entirely within Scandinavia. A practical solution was found, when the German firm Veit & Co. in Leipzig in 1889 undertook to publish the *Skandinavisches Archiv für Physiologie*. For more

than 50 years this journal has existed. In all 83 volumes and 16 supplements have appeared. They contain 1 308 contributions from 684 different authors. Only in exceptional cases have papers from Non-Scandinavians been accepted — mostly in connection with special jubilee volumes. Of the Scandinavian authors 118 are from Denmark, 191 from Finland, 22 from Norway and 276 from Sweden. The Scandinavian physiological Society, founded in 1926, has published an account of the proceedings of her congresses in the journal. Undoubtedly it has been of great value for Scandinavian physiology for half a century, and we owe the German firms Veit & Co. and Walter de Gruyter our sincere thanks for their valuable assistance.

The old idea of a physiological journal, owned by the Scandinavian physiologists and printed in Scandinavia, has never died, and the question has been taken up and discussed at numerous occasions. Recently a decision has been taken that the Skandinavisches Archiv shall be discontinued and a new journal started under the name of *Acta physiologica scandinavica*, belonging to the Scandinavian physiological Society and printed in one of the Scandinavian countries. A number of representatives of the physiological sciences in these countries have promised their collaboration. The following will act as editors: for Denmark Professor A. KROGH, for Finland Professor Y. REENPÄÄ, for Norway Professor E. LANGFELDT and for Sweden Professor G. LILJESTRAND. The journal will contain contributions to Physiology, Biochemistry and Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles will be published in English, French or German. Not more than two volumes will appear each year. In special cases supplements will appear; they will be sent to the subscribers without extra cost.

We hope that *Acta physiologica scandinavica* will promote physiology and meet with the approval of our colleagues throughout the world.

G. LILJESTRAND.

A Method of Blood Volume Determination.¹

By

L. HAHN and G. HEVESY.

(With 1 figure in the text.)

The method usually applied in the determination of blood volume is that worked out by ROWNTREE and his colleagues (1929). The principle of the method is that a dyestuff is injected intravenously and its degree of dilution determined.² As the dye only mixes with plasma, the volume of the plasma alone is thus measured. The relative volume of corpuscles and plasma is determined with the haematocrit. To arrive at the blood volume the volume of the corpuscles is added to that of the plasma.

ROWNTREE gives the following description of the method applied (comp. also FLEISCHER-HANSEN, 1928). A 1.5 per cent solution of vital red in distilled water is prepared. Four centrifuge tubes are provided and 1 c.c. of a 1.6 per cent solution of sodium oxalate is placed into each of them. A needle is inserted in the vein of one arm and 10 c.c. of blood are removed. 5 c.c. are placed into each of two centrifuge tubes for standard plasma colour. The dye is then injected. After 3 to 6 min., 10 c.c. of blood are withdrawn from the vein of the other arm and 5 c.c. placed into each of the two remaining centrifuge tubes. All four tubes are centrifuged and the relative volume of corpuscles and plasma measured. The second sample is compared with a known strength of the dye and the degree of dilution of the dye in the plasma is thus obtained.

¹ Received for publication 29 March 1940.

² Instead of a dyestuff, diphtheria antitoxin was used in some determinations (v. BEHRING, 1912; MADSEN, 1934).

When considering the possible errors of this method, the main question at issue is whether, when the second sample is collected, the dye is uniformly mixed in the plasma and none has yet escaped into the tissue spaces or urine, a further possible source of error being the adsorption of a part of the dye by the enormous surface of the capillary wall.

Determination of blood volume based on the dilution of labelled corpuscles.

In this note, we wish to describe a method of blood volume determination based on an entirely different principle from that described above. We inject into the vein of a rabbit A a known volume of labelled corpuscles taken from another rabbit B and determine the extent to which these labelled corpuscles are diluted in the circulation of rabbit A. Labelled corpuscles of rabbit B are obtained in the following way. We administer by subcutaneous injection some labelled (radioactive) sodium phosphate to rabbit B. In the course of about a week, a substantial fraction of the phosphatide molecules of the bone marrow and other organs are renewed. As this renewal takes place in the presence of labelled phosphate, the newly formed phosphatide molecules will contain labelled P atoms. Corpuscles formed in a medium containing labelled phosphatide molecules will necessarily incorporate some of them. Labelled phosphatide molecules can also enter to some extent into the corpuscles by exchange of non-active phosphatide molecules with active phosphatide molecules present in the plasma. The various ways of incorporating labelled phosphatides into corpuscles are described in detail in a paper which is in print (HAHN and HEVESY, 1940).

Besides labelled phosphatides, labelled varieties of several acid-soluble organic phosphorus compounds as, for example, those of glycerophosphate and adenosin triphosphate, are found in the corpuscles. Each of these compounds can be used as an indicator when determining the dilution of the corpuscles of rabbit B in the circulation of rabbit A. It is, however, more convenient to extract the total acid-soluble P and to use the mixture obtained as an indicator.

Determination of the blood volume of a rabbit weighing 2 kg.

a) Making use of the labelled phosphatides of the corpuscles.

We administered radioactive sodium phosphate of negligible weight having the activity of about 0.001 milliCurie to rabbit B. After the lapse of a week, 1 c.c. of blood of rabbit B containing 0.32 c.c. corpuscles was injected into the jugularis of rabbit A. After the lapse of 5 min., 50 c.c. blood were collected and, after the addition of heparin, centrifuged. The haematocrit value of this sample was found to be 0.33. The phosphatides of the corpuscles were thoroughly extracted by BLOOR's method. Their P was converted by wet ashing into phosphate. The phosphate was precipitated as ammonium magnesium salt. Before precipitation, sodium phosphate was added to the solution to obtain a precipitate of about 80 mg. The activity of the precipitate was then determined by means of a GEIGER counter. The comparison of the activity of the samples is facilitated if they have practically the same weight and it is for this reason that we added to the original sample a comparatively large amount of non-active phosphate. The corpuscles of 1 c.c. of the blood of rabbit B were also extracted with ether-alcohol and the extract treated in the way described above. The activity of the sample thus obtained was then compared with that of the corresponding sample of rabbit A.

Let us denote the injected blood volume by V_i , the volume of the sample collected for analysis from rabbit B and rabbit A, respectively, by V_I and V_{II} , and the activity of the two samples obtained by A_I and A_{II} ; then the blood volume to be determined (X) becomes

$$X = \frac{A_I \cdot V_{II} \cdot V_i}{A_{II} \cdot V_I} - V_i.$$

In some of our experiments, before injecting, for example, 1 c.c. blood into the jugularis of rabbit A, we removed 1 cc. In that case, the second term of the equation becomes 0.

Operations involved in the determination of the total blood volume are thus: measurement of the volume of three samples, and the comparison of the radioactivity of two samples.

In the above mentioned experiment the corpuscle phosphatides of 1 c.c. blood of rabbit B contained 100 relative activity units;

the activity of the corpuscle phosphatides extracted from 50 c.c. blood of rabbit A was found to be 53.3. From these values it follows that the blood volume of rabbit B amounts to $93.8 - 1 \text{ c.c.} = 92.8$.

b) Making use of labelled acid-soluble compounds of the corpuscles.

We can check the result obtained above by another procedure in which, instead of the labelled phosphatides, the labelled acid-soluble phosphorus compounds are involved. After extraction of the phosphatides, the corpuscles are extracted with 10 per cent trichloroacetic acid. The P of the filtrate obtained is converted, as described above, into ammonium magnesium phosphate. The activity of the sample obtained from rabbit A is compared with that of the sample from rabbit B, as described above. The figures obtained being 100 and 54.4 respectively, the total blood volume of rabbit A becomes

$$X = \frac{A_I \cdot V_{II} \cdot V_I}{A_{II} \cdot V_I} - V_I = \frac{100 \cdot 50 \cdot 1}{54.4 \cdot 1} - 1 = 91.9 - 1 = 90.9.$$

The use of labelled acid-soluble P compounds leads thus to practically the same result as obtained with labelled phosphatides as indicators.

The blood volume per kg of rabbit weight was found, in the experiment described above, to be 46 c.c. In another experiment the value of 38 c.c. was found.

Determination of the blood volume of a chick weighing 135 gm.

Labelled sodium phosphate was administered to chick B and, 24 hours later, 0.5 c.c. blood of this chick was injected into the jugular vein of chick A. The haematocrit values were found to be 0.26 and 0.28, respectively. We found the activity of the phosphatides extracted from the corpuscles of 1 c.c. blood of chick A to be 7.6, taking that of the phosphatides secured from the corpuscles of 1 c.c. blood of chick B to be 100. The total blood volume of chick A is thus

$$X = \frac{100 \cdot 1 \cdot 0.5}{7.6 \cdot 1} - 0.5 = 6.1,$$

or 45 c.c. per kg weight.

Loss of labelled P compounds by the corpuscles.

We wish now to discuss the possible loss of labelled P compounds by the corpuscles during the interval between the injection of labelled corpuscles of rabbit B into the circulation of rabbit A and the securing of blood samples of rabbit A. Such a loss would clearly involve a source of error by leading to too high values for the blood volume to be determined. As to the loss of labelled phosphatides, we found that the labelled phosphatide content of the corpuscles of rabbit A, after the lapse of 1 hour, amounts to 90 per cent of that found after the lapse of 6 min. This result shows that the disappearance of labelled phosphatides from the corpuscles takes place at a slow rate.

As to the disappearance of the labelled acid-soluble P molecules from the corpuscles, we find the following result. The labelled acid-soluble P content of 1 c.c. corpuscle of rabbit A, 8.5 min. after injecting the blood of rabbit B into rabbit A, is, as seen in Table 1 and Fig. 1, within the errors of experiment, identical with that found after 2.5 min.

Table 1.

Disappearance of labelled acid-soluble P compounds from the corpuscles.

Time elapsed after injecting the blood of rabbit B into rabbit A	Per cent of labelled acid-soluble P injected, present in 1 cc corpuscle of rabbit A
2.5 min.	3.39
5 '	3.21
6.5 '	3.39
8.5 '	3.30
26.5 '	3.12
60 '	2.88
120 '	2.58
210 '	1.35

We have now to investigate if, during the lapse of 5 min. or more, after which time blood samples in the experiments described in this paper were secured, a uniform mixing of the labelled corpuscles of rabbit B in the circulation of rabbit A took place. From

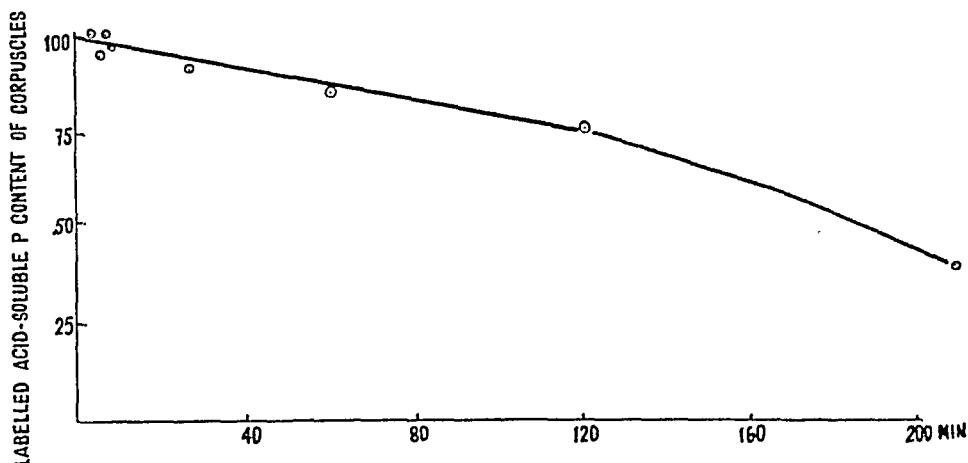


Fig. 1. Disappearance of labelled acid-soluble P from corpuscles, injected into the circulation of a rabbit.

the data contained in Table 1 and Fig. 1 we can conclude that, already after the lapse of 2.5 min., a uniform mixing actually took place. Such a result in the case of a small animal with fast circulation could be expected since, in experiments carried out on human subjects, a uniform mixing of dyestuffs injected into the plasma was found to take place in the course of 6 min. (ROWNTREE *et alia*, 1929; GRAFF *et alia*, 1931).

Discussion.

The sole difference between the normal and the labelled corpuscles is that in some of the molecules present in the normal ones P (having the mass number 31) is replaced by radioactive P (having the mass number 32). In 1 c.c. corpuscles of rabbit B, 0.06 mg phosphatide P was present. Of these 0.06 mg 10^{-11} mg were radioactive ^{32}P atoms. As 1 c.c. contains about $3 \cdot 10^9$ corpuscles, one corpuscle contains on an average 10^{-21} mg ^{32}P or only about one corpuscle in a hundred contained an active phosphatide molecule. After injecting the blood sample of rabbit B into rabbit A, a strong dilution of the labelled phosphatides took place: only one in about three thousand corpuscles now contains a radioactive phosphatide P atom. The replacement of a minute percentage of the ^{31}P atoms by ^{32}P atoms in the P compounds of the corpuscles can hardly influence to any noticeable extent the chemical properties of the corpuscles and we can, therefore, claim that, when applying the method described in this

note, no non-physiological component is introduced into the circulation. As to the β -radiation emitted by the ^{32}P atoms present in the corpuscle phosphatides, the number of β -particles emitted per minute in the total circulation of rabbit A amounts to only about 1000, while the number emitted by the total acid-soluble fraction amounts to about 30 times that figure. How insignificant these figures are can best be realized when we envisage that this radiation corresponds to that of only 10^{-9} and 10^{-8} gm radium, respectively.

When carrying out experiments as those described above on human subjects, it may be advisable to make use of the acid-soluble P compounds of the corpuscles as indicators. Since, in this case, one may use less radioactive P, such experiments can be carried out on human subjects by administering by subcutaneous injection or by mouth to the blood donor sodium phosphate having a β -radioactivity corresponding to that of about 0.01 milliCurie or even less.

Summary.

A method of blood volume determination based on the determination of the dilution of labelled corpuscles is described. Radioactive sodium phosphate is administered to rabbit B; after the lapse of some days, a known blood sample of this rabbit is injected into the vein of rabbit A. A few minutes later, the corpuscles of a known volume of the blood of rabbit A are secured, their phosphatide content extracted, and its activity measured. Corpuscles of a known blood volume of rabbit B are treated in the same way. From the ratio of the labelled phosphatide P content of the corpuscles of rabbit A and rabbit B, the total blood volume of rabbit A is calculated.

An alternative and often preferable determination is based on the comparison of the activity of the acid-soluble P secured from the corpuscle samples of rabbit A and rabbit B.

The blood volume per kg of rabbit weight is found to be 42 c.c., per kg of chick weight 45 c.c.

We wish to express our hearty thanks to Professor NIELS BOHR for putting numerous facilities at our disposal, to Professor ERNEST O. LAWRENCE for the gift of the radioactive phosphate, and to Dr. H. DAM for his effective help in the experiments with chicks.

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-

Rate of Passage of Water through Capillary and Cell Walls.

By

G. HEVESY and C. F. JACOBSEN.¹

(With 1 figure in the text).

Water molecules, which are absorbed into the circulation, will mix rapidly with those present in the plasma. They will then penetrate the capillary wall and become distributed in the extra-cellular fluid. Ultimately, they will invade the cells. Simultaneously, a loss of some of the water molecules through the kidneys, the bowels, the lungs and through peripheral evaporation will also take place. It is difficult to estimate even very roughly the rate at which some of the above processes take place. Experiments in which heavy water is used as an indicator permit, however, the determination of the rate at which individual water molecules introduced into the circulation are distributed in the body water and from these determinations to answer the above questions.

We inject a few cc. of practically pure heavy water into the jugularis of a rabbit and take at intervals blood samples from the carotis. The next step is to prepare pure water from the blood samples² and to determine its density. Let us assume that we inject 1 cc. of heavy water having a density of 1.1000, and find for the water prepared from a blood sample the value 1.001. Then we must conclude that the 1 cc. heavy water injected into the vein was diluted by 99 cc. of normal water present in the body of the rabbit in the course of the time which elapsed between the injection of the heavy water and the collection of the blood sample.

¹ Received for publication 11 April 1940.

² The total water content of the blood has to be distilled to avoid a fractionation of the diluted heavy water, the vapour pressure of deuterium oxyde being smaller than that of H₂O.

Experimental procedure.

Heavy water in contact with air containing vapour of normal water rapidly becomes lighter in consequence of the interchange in the vapour phase. In view of this interchange, it was necessary to keep the samples containing heavy water out of contact for any prolonged time with the moist atmosphere. The blood samples

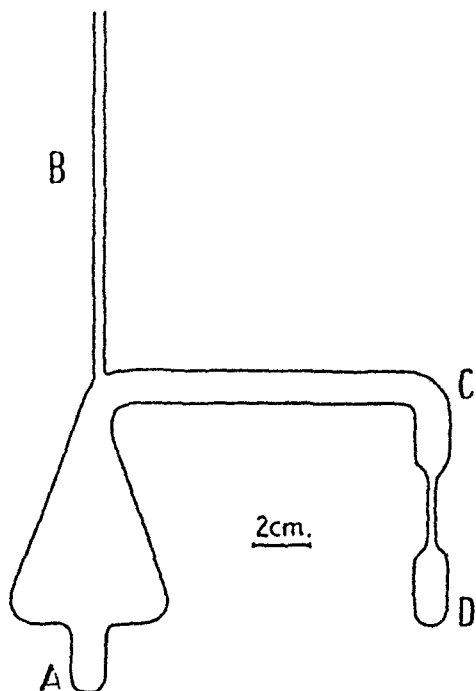


Fig. 1.

of about 1 cc. volume were collected in small dishes containing traces of heparin, the blood was transferred into part A of the glass vessel, seen in Fig. 1, and the vessel closed with soft parafin. These operations took only a few seconds. About $\frac{1}{2}$ mg. of dry P_2O_5 was added to the solution in order to neutralize any traces of ammonia present. The vessel was then cooled in the refrigerator, evacuated, and sealed off at B. Tube D was then immersed in liquid air, while the other parts of the vessel remained at room temperature. After the lapse of a few hours, the water content of the blood sample was found to be present in the form of ice in tube C. After the ice was molten and the water collected in D, this tube was sealed off. The pure water obtained by this proce-

ture was further purified by distillation in the presence of potassium permanganate and sodium peroxyde, and the density of the purified samples determined, using LINDERSTRÖM-LANG's floating drop method (LINDERSTRÖM-LANG, JACOBSEN and JOHANSEN 1938).

Experiment A.

In this experiment, a rabbit weighing 2.6 kg. was used. 9.2 cc. of heavy water having a density of 1.1049 were injected. The time which elapsed after the injection of the heavy water is recorded in the first column of Table 1, while the next column contains data on the density excess of the blood water over normal water, expressed in parts per million. (The heavy water injected had a density excess of 104 900 parts per million.) The third column contains data on the dilution of 1 cc. heavy water introduced into the circulation. In the fourth column the percentage of the weight of the rabbit which took part in the dilution process is stated.

Table 1.

Time	Density excess of blood water in parts per million	Extent of dilution of 1 cc. heavy water	Diluting water volume expressed in percentage of body weight
40 sec. . . .	1 500	644	24.7
185 sec. . . .	1 300	742	28.6
3.3 min. . .	930	1 038	40.0
6.1 min. . .	795	1 221	46.9
24.1 min. . .	527	1 825	70.2
5 days . . .	339	2 843	109
39 days . . .	89	10 810	417

The injection lasted 40 sec.; the time recorded in column 1 is calculated from the moment half of the water was injected; the first blood sample was collected 20 sec. after all the heavy water was injected. In the course of such a short time as 40 sec., 644 cc. of the body water took part in diluting the heavy water injected and, after the lapse of 24 min., as much as 1 825 cc.

The plasma water cannot diffuse into the cells without passing the capillary wall. If the last mentioned process took place within 40 sec., then the volume of the diluting water should be at least equal to that of the extracellular fluid which amounts, in a rabbit

weighing 2.6 kg, to 670 cc¹. As seen in column 4, the volume of the diluting water was only slightly less, namely 650 cc., than this value. The total water content of the rabbit amounts to 70 to 75 per cent of its weight, corresponding to a volume of 1820 to 1 950 cc. As seen in column 4, the sample collected after the lapse of 24 min. was found to be diluted by 1825 cc. body water. Within that time, therefore, a distribution of the heavy water in almost the total body water took place, though some of the water present in certain organs may not have taken part in the exchange process². This point can only be settled by investigating the density of tissue water. The above figures suggest that, in contradistinction to a very fast invasion of the interspaces, the penetration into the cells is a somewhat slower process.

In the samples collected shortly after the start of the experiment the dilution, due to a loss of heavy water by the body, can be disregarded. This is not the case in experiments lasting several hours or days. In the course of 5 days, for example, the loss of water through the kidneys alone amounts to about 1 liter, thus to 38 per cent of the rabbit's weight. In this case, the excretion of a corresponding part of the heavy water is responsible for the at first sight puzzling value of 109 per cent found. The hydrogen atoms bound to oxygen or nitrogen in the various organic compounds present in the body exchange speedily with those present in the water or heavy water molecules, and this process will also increase the dilution figures observed, as a removal of deuterium acts in the same way on the water density figures as does dilution by normal water. In view, however, of the fact that the amount of hydrogen present in the organic compounds is small compared with that of the hydrogen incorporated in water molecules, the process mentioned above will not much influence the dilution figures obtained. In experiments of long duration, a successive replacement of most the hydrogen atoms present in organic molecules will take place, giving an additional outlet to some of the deuterium atoms present in the body water. The percentage of hydrogen present in the fats of the body which exchanges within 1 hour with water hydrogen is negligible; the corresponding amount of protein hydrogen is not (USSING 1938). The water equivalent of this hydrogen amounts, however, only to $\frac{1}{2}$ to 2 per cent of the body weight, or 13 to 52 cc. in the case of rabbit A and 8 to 30 cc.

¹ Comp., for example, A. KROGH (1937).

² Comp. USSING (1938).

in the case of rabbit B. The amount of catabolic water formed in the course of 1 hour in the rabbits amounts only to about 0.1 per cent of the body weight. As seen in Table 1, after the lapse of 39 days, the density excess of the blood water very much declined. This decline is mainly due to loss of the heavy water and, thus, of a corresponding amount of normal water present at the start of the experiment from the body. About 5/6 of these molecules was lost in the course of 39 days. In the case of human subjects, who drank heavy water, it was found (HEVESY and HOFER, 1934) that, in the course of 9 days, half of the heavy water taken was lost.

In the above connection, it is of interest to recall the experiments carried out by McDOUGALL, VERZAR, ERLÉNMEYER and GAERTNER (1934). They injected a solution of heavy water into the jejunal loops of rats and investigated the heavy water content of the intestinal fluid of the rats killed 1 hour after the start of the experiment. They found the diluting water volume of the rat to amount to 66 per cent of the body weight.

Experiment B.

This experiment was carried out on a rabbit weighing 1.5 kg. 5.0 cc. of heavy water were injected. The injection took 6 sec. The first blood sample was taken in the interval of 22—26 sec. after the start of the experiment. The time recorded is reckoned from the moment that half of the heavy water was injected until half of the blood sample was collected.

Table 2.

Time	Density excess of blood water in parts per million	Extent of dilution of 1 cc. heavy water in the circulation	Diluting water volume expressed in percentage of body weight
21 sec. . . .	1 034	506	34
80 sec. . . .	856	612	40.8
1.8 min. . . .	794	661	44.1
3 min. . . .	719	727	47.8
5.2 min. . . .	570	921	61.4
7.9 min. . . .	495	1 056	70.4
13 min. . . .	490	1 070	71.3
22.2 min. . . .	440	1 190	79.4
30 min. . . .	450	1 167	77.9
48 hours . .	412	1 274	85

While rabbit A had not shown any sign of distress after the blood samples were taken, this was not the case with rabbit B. In the course of the two days following the start of the experiment, only a small amount of urine was produced by rabbit B, with the consequence that no pronounced effect due to loss of heavy water through the kidneys is shown by the density figures of the blood water sample of rabbit B collected after the lapse of 2 days, in contradistinction to the results obtained when investigating the blood water of rabbit A after the lapse of some days.

Discussion.

We found that, within about $\frac{1}{2}$ min., heavy water injected into the jugularis of rabbits was diluted by a large amount of body water, the volume of which corresponds to about that of the extracellular space of the body. This rapid dilution is followed by a second, slower process, presumably due mainly to a further dilution of the heavy water by cellular body water. From these findings it follows that all water molecules present in the plasma pass with a very high speed through the capillary walls and with a slower, but still remarkable speed through the cell walls, and vice versa. To this conclusion one may possibly object that heavy water (D_2O) may show a different behaviour from H_2O and recall the results obtained in the investigation of the rate of hemolysis of erythrocytes of cattle and rats, which was found to take place about 44 per cent more slowly in D_2O than in H_2O (PARPART, 1935; BROOKS, 1935).

Contrary to those of the above mentioned authors, our experiments were not carried out with pure D_2O but with very diluted heavy water, the viscosity and other properties of which only slightly differ from those of H_2O . Our most concentrated samples contained, in fact, less than 2 per cent D_2O , most of them containing very much less. The D_2O injected into the vein is diluted at once. The above mentioned authors did not use heavy water as an indicator for water; they were interested in the differences shown by H_2O and D_2O when penetrating into corpuscles. When heavy water is used as an indicator, it should always be used in a state as diluted as possible, partly for the above reasons and partly because such diluted solutions contain mainly DHO which is very similar to H_2O , while D_2O is much less so.

In view of the high speed of capillary passage found in our experiments, it is of interest to calculate the time taken by the diffusion of water molecules through the capillary spaces. The mean displacement τ of water in water is $\sqrt{2D}$ (where D denotes the diffusion constant of water in water, determined by using heavy water as an indicator), is 2 cm per day (ORR and THOMSON, 1935). Taking the size of the capillary vessel as $20\ \mu$, we arrive at the result that the displacement of water molecules within that space takes 1×10^{-6} day, or about 1/10 sec., thus an exceedingly short time.

Summary.

Heavy water is injected into the vein of rabbits and blood samples taken at intervals from the artery. The density of the water prepared from the blood samples is determined and, from the density difference between the injected heavy water and the blood water, the extent of dilution, which the heavy water molecules experienced in the body at different times, calculated.

As soon as a $\frac{1}{2}$ min. after the injection, a dilution of the heavy water by an amount of body water corresponding in volume to about that of the extracellular space of the body, is found. This very rapid rate of dilution is followed by a somewhat slower dilution process, in which presumably the cellular water participates.

After the lapse of less than $\frac{1}{2}$ hour, the heavy water molecules are evenly distributed over almost the total body water.

After the lapse of 39 days, only about $\frac{1}{5}$ of the heavy water injected is still present in the body.

There is no reason to assume that the heavy water (mainly DHO) molecules show a markedly different behaviour from that of the normal water (H_2O) molecules present in the body, and we have, therefore, to conclude that within about $\frac{1}{2}$ min. a sufficient flow of water takes place through the capillary walls to lead to an almost perfect mixing of the blood and the interspace water. An analogous interaction between cellular and extracellular water takes less than $\frac{1}{2}$ hour.

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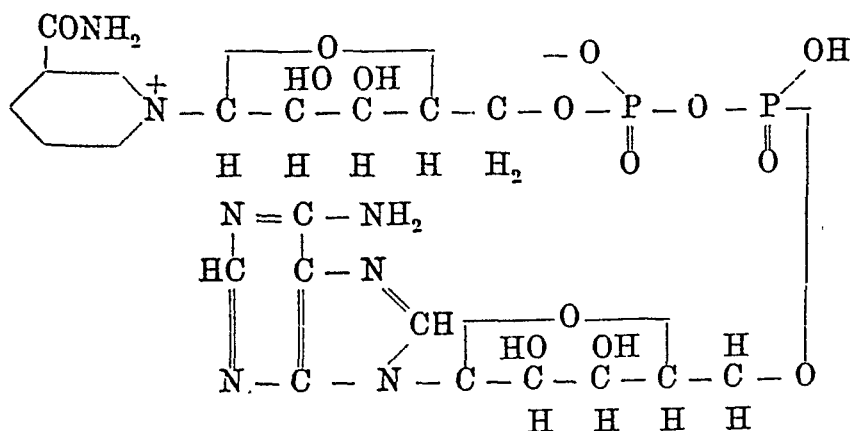
Zur Pharmakologie der Cozymase.¹

Von

H. v. EULER, U. S. v. EULER und F. SCHLENK.

Mit 5 Figuren im Text.

Die Cozymase, welche 1936 in fester Form dargestellt wurde (H. v. EULER, ALBERS u. SCHLENK), hat die Zusammensetzung $C_{21}H_{27}O_{14}N_7P_2$ und wurde als Adenin-Nicotinsäureamid-dinucleotid erkannt (SCHLENK u. H. v. EULER, 1936). Ihre Konstitution ist nunmehr durch die Versuche von SCHLENK (1936) auch in ihren Einzelheiten aufgeklärt², sie wird durch folgende Formel beschrieben:



Diese Formel stützt sich auf den Nachweis, dass die Cozymase einbasisch reagiert sowie auf die Isolierung und Charakterisierung von Spaltprodukten, nämlich

¹ Der Redaktion am 3. Mai 1940 zugegangen.

² Die (ausserordentlich wahrscheinliche) Annahme, dass die Pentose des Nicotinsäureamid-nucleosides Ribose ist, soll demnächst noch besonders bestätigt werden.

1) die Charakterisierung der Pentose-Phosphorsäure, welche von SCHLENK (1936) isoliert und von KARRER (H. v. EULER, KARRER u. BECKER, 1936) als Pentose-5-phosphorsäure erkannt wurde.

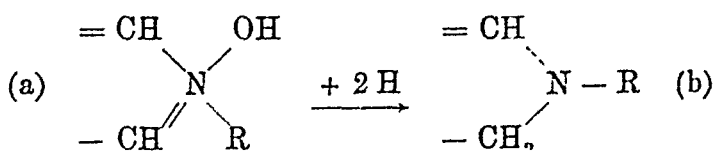
2) auf die Isolierung eines von VESTIN und H. v. EULER (1936) dargestellten leicht hydrolysierbaren Körper, nach VESTIN u. Mitarb. (1937) Adenosin-5-pyrophosphorsäure und

3) auf die Isolierung des Nicotinsäureamid-nucleosides (SCHLENK, 1938).

Cozymase kommt in fast allen Zellen als unentbehrlicher Bestandteil vor. Sie ist notwendig für den Kohlenhydratabbau als Wasserstoffüberträger (H. v. EULER, ADLER u. HELLSTRÖM, 1935) und zwar als Codehydrase bei enzymatischen Teilreaktionen der Atmung, Glykolyse und Gärung, in erster Linie für die diesen Vorgängen gemeinsame Reaktion:

Triosephosphorsäure + Co \rightleftharpoons Phosphoglycerinsäure + CoH₂, wobei die Cozymase, Co, in Dihydrocozymase, CoH₂, übergeführt wird.

Wie die Modellversuche von KARRER (KARRER u. MITARB., 1937) gezeigt haben, ist die wasserstoffübertragende Fähigkeit der Cozymase an die Pyridinium-Form gebunden, also an das Vorkommen einer Gruppierung (a), welche in (b) übergeht:



Die zweite Nucleotid-Komponente, die Muskeladenylsäure (= Adenosin-5-phosphorsäure), geht, wie die obige Formel zeigt, in das Molekül der Cozymase ein.

Zur Pharmakologie der Cozymase.

Über die pharmakodynamischen Wirkungen der Cozymase liegen bereits Untersuchungen vor, die aber mit teilweise unreinen Präparaten ausgeführt worden sind. GARD (1931) fand, dass ein Cozymasepräparat von etwa ACo 100,000 den Blutdruck des Kaninchens senkte und die Koronargefäßdurchströmung am überlebenden Herzen erhöhte. Die Wirkungen waren auch nach Inaktivierung der Cozymase vorhanden. U. S. v. EULER und

GADDUM (1931) unterwarfen ein Cozymasepräparat von etwa demselben Reinheitsgrad einer etwas eingehenderen Prüfung und verglichen dabei die Wirkung von Cozymase mit der von reinem Adenosin mit Hinblick auf die schon damals vermutete Beziehung der Cozymase zur Adenylsäure. Sie fanden, dass die Cozymase etwa dieselben qualitativen Wirkungen auf Blutdruck und isolierten Darm des Kaninchens sowie auf isolierten Meerschweinchenuterus wie Adenosin zeigte. Kurz vorher hatten DRURY und SZENT-GYÖRGYI (1929) auf die pharmakodynamischen Wirkungen der Adenylsäure und des Adenosins aufmerksam gemacht. In quantitativer Hinsicht verhielten sich die Wirkungen von Cozymase und Adenosin wie etwa 2 : 3, was mit den verschiedenen Molekulargewichten in Beziehung gebracht wurde.

Mit einem reinen Cozymasepräparat haben wir die Wirkungen am Herz, Blutdruck und Atmung des Kaninchens, sowie den Effekt am isolierten Kaninchendarm und Meerschweinchenuterus studiert.

a) Wirkung auf Herzfrequenz, Blutdruck und Atmung des Kaninchens.

In Mengen von 0.2—2 mg der reinen Substanz ergab sich nach intravenöser Darreichung eine reine Drucksenkung wie in den Abbildungen 1 und 2 zu ersehen ist. Im Vergleich mit Adenosin (Brit. Drug Houses) war die Wirkung der Cozymase etwas abweichend, was sich einerseits in einer langsamer einsetzenden Senkung und in einem kleineren Betrag derselben darstellte. Mit gleich grossen Mengen von Cozymase und Adenosin (1 mg) betrug die maximale Drucksenkung des ersten etwa 30 % in 15 Sekunden, während die entsprechende Wirkung von Adenosin 40 % in 10 Sekunden war. Die Herzfrequenz nahm mit beiden Substanzen beträchtlich ab, von 340 bis 280 pro Minute nach 1 mg Cozymase und von 340 bis 250 nach 1 mg Adenosin in einem Falle. Das Minimum der Herzfrequenz lag zwischen etwa 5 und 15 Sekunden nach der Injektion und die angeführten Zahlen beziehen sich auf diese Periode.

Die Atmungsbewegungen wurden in den Blutdruckversuchen registriert und zeigten regelmässig eine Zunahme von Amplitude und Frequenz, während der Drucksenkung. Es erscheint jedoch zweifelhaft ob diese Wirkung spezifisch ist; sie kann auch eine Folge der Drucksenkung sein.

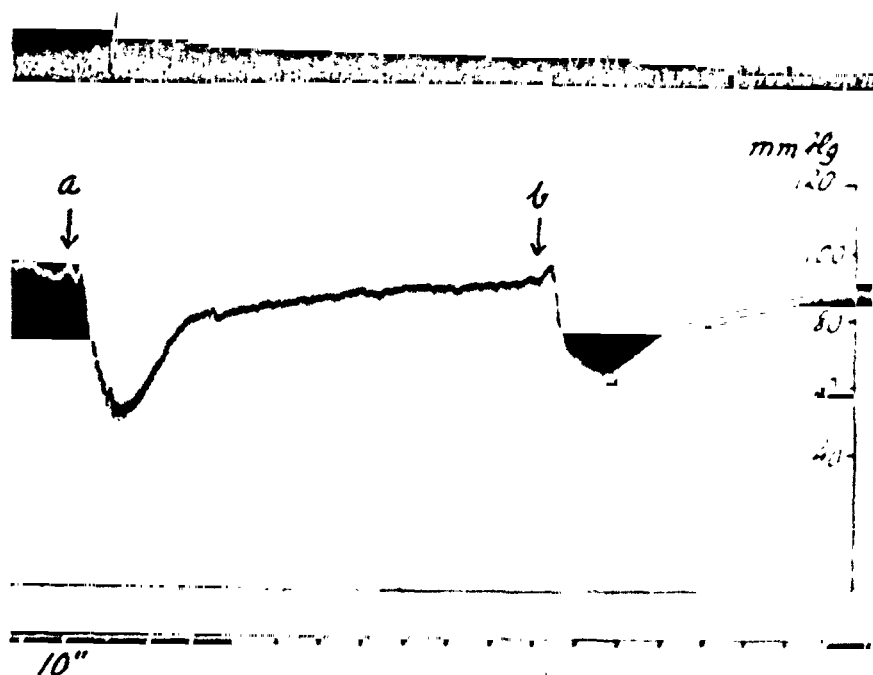


Abb. 1. Blutdruck. Kaninchen. Urethan. Obere Kurve Atmungsbewegungen.
Bei a 1 mg Adenosin, bei b 1 mg Cozymase intravenös.

Die Blutdrucksenkung nach Adenosin und Adenylverbindungen ist teils auf eine Herzwirkung und teils auf eine Gefäßwirkung zurückzuführen; bei der letzteren dürfte sowohl eine Konstriktion der Lungengefäße wie eine Dilatation der Arteriolen in mehreren Gefäßgebieten mitwirken (DRURY u. SZENT-GYÖRGYI, 1929; BENNET u. DRURY, 1931; DRURY, 1932; ZIPF u. GIESE, 1933). Die erstgenannten Autoren geben auch an, dass die Wirkung mit der Desaminierbarkeit in Beziehung steht, denn Hefeadenylsäure wie Guanylsäure waren inaktiv, und diese Verbindungen werden nach SCHMIDT (1928) nicht oder nur langsam desaminiert. In späteren Untersuchungen hat es sich herausgestellt, dass Hefeadenylsäure eine Blutdruckwirkung besitzt (BENNET u. DRURY; EULER, 1933); diese ist jedoch von derjenigen der Muskeladenylsäure oder des Adenosins recht verschieden und verläuft viel langsamer. Die auch von EMBDEN (1932) gehegte Auffassung, dass die

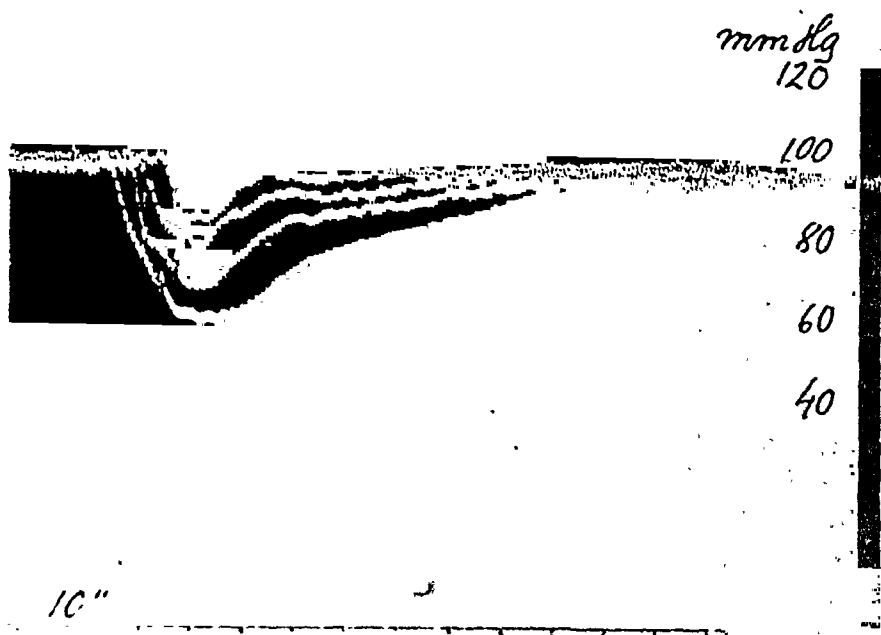


Abb. 2. Blutdruck, Kaninchen, Urethan. Die verschiedenen Blutdruckkurven zeigen die Wirkungen von 0.2, 0.5, 1 und 2 mg Cozymase intravenös.

Desaminierbarkeit von grosser Bedeutung für die Aktivität ist, wird von PARNAS und OSTERN (1932) bestritten.

Wir haben die Blutdruckwirkung von einigen Adeninderivaten im Kaninchenversuch geprüft (Abb. 3). Hieraus ergibt sich dass Inosinsäure und Desaminocozymase völlig unwirksam waren, während Hefeadenylsäure, Adenosin und Adenosintriphosphorsäure, wie schon früher bekannt war, blutdrucksenkend wirkten. Bedeutende quantitative Verschiedenheiten in der Wirkung waren zu verzeichnen: somit war die Wirkung von Adenosin, auf gleich grosser Menge bezogen, viel stärker als die der Hefeadenylsäure, und Adenosintriphosphorsäure noch bedeutend wirksamer als das Adenosin. Besonders auffällig ist der Unterschied in der Wirkung zwischen Hefeadenylsäure und Adenosin, die viel ausgesprochener ist als in den entsprechenden Versuchen von BENNET und DRURY. Es liegt die Vermutung nahe, dass die Verschiedenheit der Wirkung mit der Zusammensetzung der Präparate in Beziehung steht. Das in unseren Versuchen verwendete Adenosin stammte von British Drug Houses und die Hefeadenylsäure von Boehringer u. Söhne. Die Desaminocozymase (SCHLENK, HELLSTRÖM u.

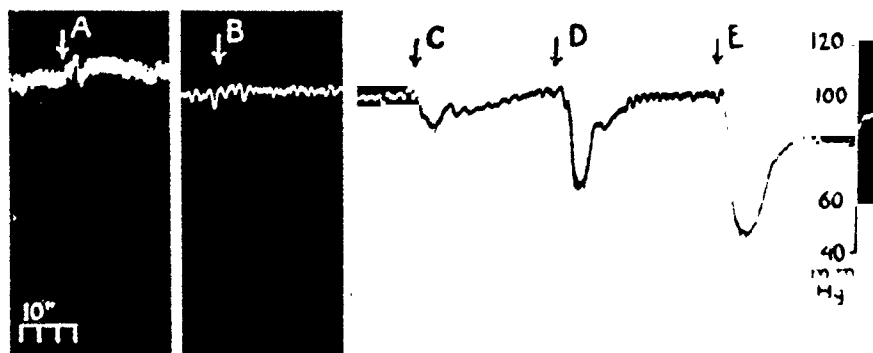


Abb. 3. Blutdruck, Kaninchen, Urethan. A. 2 mg Desaminocozymase, B. 1 mg Inosinsäure, C. 0.5 mg Hefeadenylsäure, D. 0.5 mg Adenosin, E. 0.5 mg Adenosintriphosphorsäure, intravenös.

H. v. EULER, 1938) in welcher die NH_2 -Gruppe des Adeninrestes, wie in der Inosinsäure, durch OH ersetzt ist, wurde von F. SCHLENK dargestellt.

Unsere Versuche haben eine weitere Stütze für die von DRURY u. SZENT-GYÖRGYI und EMBDEN ausgesprochene Meinung bezüglich der Bedeutung der Desaminierbarkeit geliefert, wenn auch andere Erklärungsgründe für die Verschiedenheit der Wirkung in Betracht kommen können.

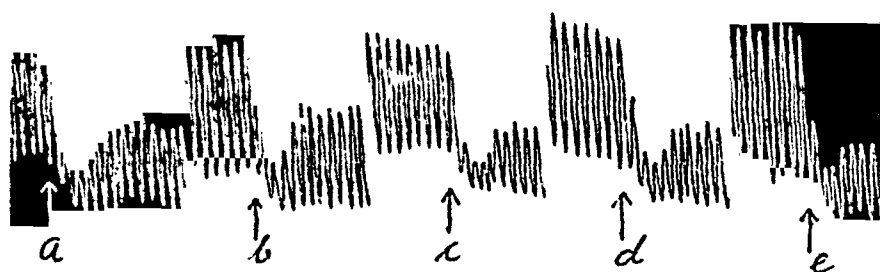
b) Wirkung an isolierten Organen.

Am isolierten Kaninchendarm wirkt die reine Cozymase hemmend in einer ähnlichen Weise wie das Adenosin. Ein quantitativer Unterschied zwischen den beiden Substanzen bestand auch hier, indem die Wirkung von Cozymase in einer Konzentration von 1 : 150,000 schwächer war als die von Adenosin in gleicher Konzentration. (Abb. 4 A.)

Während keine der beiden Substanzen in einer Konzentration von 1 : 30,000 auf den isolierten Kaninchenuterus wirksam war, konnte mit beiden eine Kontraktion des Meerschweinchenuterus erzielt werden. In diesem Falle waren beide Substanzen etwa gleich stark wirksam, wie aus der Abb. 4 B hervorgeht. Nach Versuchen von DEUTICKE (1932) steigt die Uteruswirksamkeit mit steigender Phosphorylierung des Adenosins.

Ausser der Cozymase haben wir ebenfalls Desaminocozymase, Inosinsäure, Hefeadenylsäure und Adenosintriphosphorsäure am

A



B

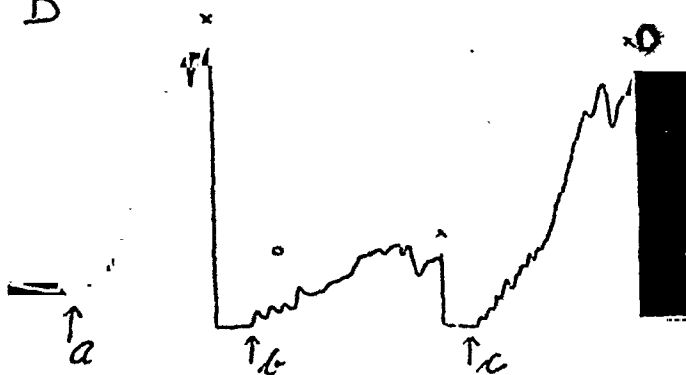


Abb. 4. A. Isolierter Kaninchendarm. a, c, und e Adenosin 1 : 150,000, b. Cozymase 1 : 150,000, d Cozymase 1 : 60,000. B. Isolierter Meerschweinchenuterus. a Cozymase 1 : 60,000, b. Adenosin 1 : 150,000, c Adenosin 1 : 60,000.

Kaninchendarm geprüft. Abb. 5 zeigt, dass auch hier die Wirkung der Hefeadenylsäure weit hinter der des Adenosins steht. Dagegen war die Wirkung der Adenosintriphosphorsäure — im Gegensatz zur Blutdruckwirkung — schwächer als die des Adenosins. Inosinsäure, wie auch Desaminocozymase waren in den verwendeten Mengen unwirksam.

Zusammenfassend lässt sich sagen, dass die reine Cozymase in grossen Zügen die charakteristischen Wirkungen einer Adenylver-

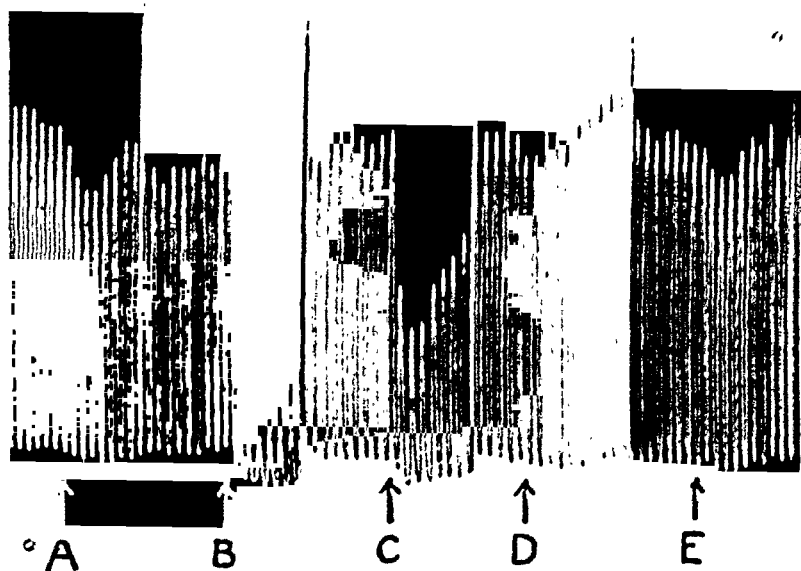


Abb. 5. Isoliertes Dünndarmstück, Kaninchen, Badvolumen 30 ccm. A. 0.5 mg Hefeadenylsäure, B. 0.5 mg Adenosin, C. 0.5 mg Adenosintriphosphorsäure, D. 0.5 mg Desaminocozymase, E. 0.5 mg Inosinsäure.

bindung zeigt, wenn auch gewisse Verschiedenheiten sowohl in qualitativer wie quantitativer Richtung beobachtet werden konnten.

Besprechung.

Adenylsäure und Adenosin-triphosphorsäure (wie auch Adenosin-diphosphorsäure) spielen eine ausserordentlich wichtige Rolle als Phosphatüberträger (LOHMANN, 1931). Cozymase selbst ist als Phosphatüberträger in Betracht gezogen worden (auf die diesbzgl. Diskussion soll hier nicht eingegangen werden) (LOHMANN u. MEYERHOF, 1934). Cozymase steht jedenfalls der Adenylsäure in dieser Hinsicht an Wirkungsfähigkeit nach. Man hat gelegentlich angenommen, dass ein bewegliches enzymatisches Gleichgewicht statthat:

Cozymase (Dihydrocozymase) \rightleftharpoons Nicotinsäureamid-nucleotid + Adenylsäure. Dafür, dass ein solches bewegliches Gleichgewicht eine physiologische Rolle, im Organismus spielt, fehlen aber bis jetzt ausreichende Anhaltspunkte; man wird für die Koppelung

zwischen Wasserstoffübertragung und Umphosphorylierung bei der Glykolyse eine andere Erklärung suchen müssen.

Für die Beurteilung der pharmakologischen Wirkungen der Cozymase, der Adenylsäure, Adenosintriphosphorsäure und des Adenosins sind folgende Tatsachen in erster Linie in Betracht zu ziehen.

a) Adenosin und Cozymase zeigen angenähert die gleiche blutdrucksenkende Wirkung am Kaninchen und hemmende Wirkung am isolierten Kaninchendarm. Die Blutdruckwirkung der Adenosin-triphosphorsäure übertrifft diejenige der Cozymase und der anderen Adenosinderivate.

b) Adenin ist wirkungslos, ebenso Desaminocozymase, Inosinsäure, Guanylsäure und Nukleinsäure.

c) Hefeadenylsäure (Adenosin-3-phosphorsäure) erwies sich bei unseren Versuchen (im Gegensatz zu denen von PARNAS und OSTERN, 1933) als weniger wirksam. Die gefundene schwache Wirksamkeit dürfte darauf zurückzuführen sein, dass die Hefeadenylsäure beim Versuch dephosphoryliert wird, worauf Adenosin zur Wirkung kommt.

Die Phosphorylierung des Adenosins verläuft, soviel bekannt ist, im Tierkörper rasch; das gleiche gilt von der Spaltung der Cozymase. (H. v. EULER u. HEIWINKEL, 1937; H. v. EULER, SCHLENK, HEIWINKEL u. HÖGBERG 1938). Das System Adenylsäure-Adenosin-diphosphorsäure und Adenosin-triphosphorsäure liefert das Coenzym der Phosphorylierung und ist dadurch mit dem anaeroben und aeroben Kohlenhydratabbau verknüpft.

Die hier gefundenen und erwähnten Tatsachen berechtigen zur Annahme, dass Cozymase wie auch Adenosin und dessen blutdrucksenkenden Derivate in dem Masse wirken, als sie durch Spaltung bzw. Synthese in Cophosphorylasen übergehen und so den Phosphatstoffwechsel beschleunigen.

Zusammenfassung.

1. Reine Cozymase (Adenin-nikotinsäureamid-dinukleotid) besitzt annähernd die gleiche blutdrucksenkende, darmhemmende und uteruserregende Wirkung wie Adenosin. Desaminocozymase ist in entsprechenden Mengen unwirksam.

2. Hefeadenylsäure war in unseren Versuchen weniger wirksam als Adenosin, das seinerseits schwächer als Adenosin-triphosphorsäure wirkte.

3. Die Wirkung der Cozymase und der Adenylverbindungen wird mit ihrer Bedeutung für den Phosphatstoffwechsel in Beziehung gesetzt.

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Studies in Glycoproteins.¹

By

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(With 2 figures in the text.)

In the past glycoproteins have usually been defined as conjugated proteins in which a simple protein is combined with a carbohydrate compound other than nucleic acid. As in later years simple proteins such as albumins and globulins have been found also to contain a carbohydrate group, this definition has become unsatisfactory. Between the proteins hitherto called simple proteins and those comprised under the name glycoproteins there exists, however, a certain *quantitative* difference with regard to the carbohydrate group. The former contain at most a few per cent of carbohydrate, whereas the carbohydrate content of the latter amounts to 10—20 per cent or even more. This fact justifies the keeping of the name glycoproteins for the substances until now grouped under this name. It must be remembered, however, that no sharp differentiation between simple proteins and glycoproteins can be based on the difference just pointed out, since some proteins have recently been found which contain 6—10 per cent of carbohydrate.

There is a small group of glycoproteins which contain a *neutral* carbohydrate component of the same type as that of the simple proteins, i. e. a polymerised dihexose-hexosamine. The ovomucoid and the seromucoid are the best known members of this group. It is worthy of notice that these substances differ from all other glycoproteins in that they are not precipitated from their neutral solutions on the addition of acetic acid. — It is generally believed that the carbohydrate in these mucoids as well as that in simple proteins is firmly bound to the protein. This opinion

¹ Received for publication 7 May 1940.

appears to rest almost exclusively on the fact that an isolation of the carbohydrate component has not been possible to attain without a far-going disintegration of the protein. Although not quite conclusive this fact no doubt gives strong support to the opinion mentioned. Direct evidence of the ovomucoid being a chemical unit and not a mixture of carbohydrate and protein was recently given by HESSELVİK (1938) in this laboratory, who found this protein to be electrophoretically well-defined and quite uniform.

With the exception of the mucoids of the group just mentioned all other glycoproteins seem to contain an *acid* carbohydrate. This is true for the *mucins*, such as submaxillary and synovial mucin, as well as for *tissue mucoids*, e. g. chondromucoid, tendomucoid and hyalomucoid. The nature of the carbohydrate group in these substances is not in all instances clear. For a long time the generally accepted view of the carbohydrate component was that of LEVENE (1925): that it was constituted either of chondroitin sulphuric acid or mucoitin sulphuric acid. The chondromucoid no doubt contains chondroitin sulphuric acid. The same carbohydrate acid seems also to be present in all mucoids of the connective tissues, including those of bone and umbilical cord (MEYER 1938). The mucoitin sulphuric acid, on the other hand, has actually never been demonstrated as being the main carbohydrate component in any isolated glycoprotein. LEVENE and his co-workers (1925) prepared mucoitin sulphuric acid from various materials containing glycoproteins such as gastric mucosa and cornea but not from isolated glycoproteins. Glycoproteins in these materials are rich in carbohydrate but contain so little sulphuric acid, that mucoitin sulphuric acid, if actually present, probably ought to be regarded more as an impurity than an integral part of the protein (BLIX et al. (1935), BLIX (1936), KARLBERG (1936)). Ester bound sulphuric acid has, in fact, never been found in more than negligible amounts in other glycoproteins than those containing chondroitin sulphuric acid, i. e. chondromucoid, tendomucoid etc.

Two new acid carbohydrates, both sulphur-free, have in later years been demonstrated as components of glycoproteins. One of them was first prepared from vitreous body and therefore named *hyaluronic acid*. According to MEYER et al. (1934, 1936) it is composed of equimolar parts of glucuronic acid, glucosamine and acetic acid, and might therefore have been formed from mucoitin

sulphuric acid through splitting off the sulphuric acid. As hyaluronic acid can be obtained in the pure state from different materials employing only very mild procedures, it is, however, no doubt pre-formed in the living tissues. Besides in the vitreous body the hyaluronic acid has been found in synovial mucin, in umbilical cord and in aqueous humor (MEYER (1938)). — In submaxillary mucin BLIX (1936) found a carbohydrate acid, composed of hexosamine, acetic acid and a hydroxacid. The nature of the latter has not yet been elucidated. Possibly it might be a desoxyhexuronic acid (BLIX (1938)). This carbohydrate thus seems to be chemically closely related to the hyaluronic acid.

As the protein component in the glycoproteins containing an acid carbohydrate can be supposed to contain, just as other simple proteins, some neutral carbohydrate, these glycoproteins should indeed contain *two* different carbohydrates, namely, in addition to the main, i. e. the acid one, a few per cent of a neutral carbohydrate. This has actually been demonstrated for the submaxillary (BLIX (1936)) and for the synovial mucin (MEYER et al. (1939)).

Concerning the relations between the carbohydrate acids and the proteins accompanying them in living materials the knowledge is still very incomplete. MÖRNER (1889) and SCHMIEDEBERG (1891) were of the opinion that the chondroitin sulphuric acid was partly present as alkali salt in the cartilage. MÖRNER (1889) showed that chondroitin sulphuric acid forms precipitates with simple proteins when neutral solutions of these substances are acidified with acetic acid. Such precipitates are generally formed between chondroitin sulphuric acid and proteins on the acid side of the isoelectric point of the latter, that is, between the carbohydrate acid and protein cations. The same appears to be true for the hyaluronic acid (MEYER et al. (1936)). As the routine method for the preparation of most glycoproteins containing carbohydrate acid consists of extraction under neutral or faintly alkaline conditions followed by precipitation by addition of acetic acid, it is tempting to think that the glycoproteins thus precipitated are *artificial* products, the components existing uncombined with each other in the tissues as well as in the neutral or alkaline extracts, combining only when protein cations are created on acidification. Only with a basic protein such as histone should the carbohydrate acid be able to combine in this way at hydrogen ion concentrations within the physiological range.

The supposition that the chondroproteids should be regarded as salts between carbohydrate acid and protein was put forward many years ago (SCHMIEDEBERG (1891), TAKAHATA (1924)).

MEYER et al. (1937, 1) have found the complexes formed between chondroitin sulphuric acid and simple proteins remarkably constant in spite of wide variation of the relative amounts of the reactants used. The acidbinding capacity of the protein component was found to be about the same when calculated from the carbohydrate-protein complex (taking the carbohydrate as a dibasic acid) as when calculated from compounds between protein and acid dyes or from the basic amino acid content of the protein. The ratio between hexosamine and nitrogen in native cartilage was found to be about the same as in artificial chondroitin sulphuric acid-gelatin complexes. MEYER et al. (1937, 2) therefore consider "the major part" of the cartilage to be a protein salt of chondroitin sulphuric acid.

Recently MEYER (1938) suggested that also the glycoproteins derived from vitreous humor, umbilical cord and synovial fluid are to be regarded as salts between polysaccharide acid and proteins and that in the native materials the hyaluronic acid exists "largely in dissociated form". This view appears to be based chiefly on the facts that the hyaluronic acid can be isolated from the mentioned materials without the use of hydrolytic means and that the artificial glycoproteins prepared from hyaluronic acid and simple proteins are very similar in composition and properties to the glycoproteins obtained from various natural sources. — MEYER even proposes that the term "mucin" should be used "only in a physiological sense to denote a viscous fluid of secretory origin".

It is clear that if compounds between proteins and carbohydrate acids exist in living tissues, the binding between the components must be a loose one, at least in the instances where they have been separated without hydrolytic procedures. The polysaccharide acids might exist in salt-like combination with basic proteins *intra vitam*, but as yet practically nothing is known about the electrochemical properties of the protein in the materials in question. It should not be overlooked that loose complexes of another kind between protein and carbohydrate acid may also occur. Direct experimental evidence of the relations between protein and carbohydrate acid in native materials is obviously still very limited.

The aim of the present investigations has been to find out by means of electrophoretic analyses if, in solutions of some typical glycoproteins containing a *polysaccharide acid*, or in native material holding such glycoproteins, the carbohydrate exists free or in combination with protein. The glycoproteins chosen for this investigation were the following: hyalomucoid, synovial mucin and submaxillary mucin. In addition a few experiments were conducted on cartilage extracts.

Before entering upon a description of the experiments performed it is necessary to give a short account of some electrophoretic investigations recently made on vitreous body and on synovial fluid.

HESSELVİK (1938) submitted filtered vitreous bodies to electrophoresis in the apparatus of TISELIUS. Three distinct boundaries regularly appeared, the mobilities of which were determined at various pH values. The fastest migrating boundary (u about $12 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \times 10^5$ at pH 7.5) disappeared if the mucoid was removed before the electrophoresis by precipitation with acetic acid. The electrophoretic mobilities of the other two components were of the same order as those of albumin and γ -globulin of blood serum. It was concluded that the fastest migrating component was the hyalomucoid. As, however, this component was not isolated and analysed, it might have been free hyaluronic acid. The synovial fluid has been electrophoretically studied by HESSELVİK (1940). It shows four distinct boundaries. The fastest boundary (u about $11 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \times 10^5$ at pH 7.5) disappears if the mucin is removed by precipitation with the aid of acetic acid. The mobility values of the other three correspond to those of serum albumin and serum globulin β and γ respectively.

The submaxillary mucin has, as far as I know, not been previously investigated electrophoretically, nor have cartilage extracts.

Experimental.

The electrophoresis method recently described by TISELIUS (1937) was employed. The glycoprotein solutions were dialysed before the electrophoresis against a phosphate buffer of pH 8.03 and of a ionic strength of 0.1. The same buffer was used as electrode solution. The electrophoreses were carried out at $\pm 0^\circ$ and with a potential gradient of about 5 V/cm. — The nitrogen

determinations were made with the micro KJELDAHL method. Hexosamine was determined by the method of ELSON and MORGAN as modified by NILSSON (1936). 2 ml. of the solutions analysed were heated for 12 hrs. with 5 ml. 2 *N* HCl on the boiling water bath before the hexosamine determination.

1. Experiments with vitreous body and with hyalomucoid. —

500 ml. filtered vitreous bodies from cattle were dialysed against water, concentrated at room temperature to 100 ml., filtered again and dialysed overnight against the buffer used in the electrophoresis experiments. In agreement with earlier findings, three boundaries appeared on electrophoresis. The fastest migrating component was isolated in a series of experiments. The yields from all experiments were collected and the solution (28 ml.) after concentration to half the volume, submitted to a new electrophoresis. The second electrophoresis was performed in order to free the solution from traces of the other components which might have been present on account of a not quite perfect separation. On this occasion two boundaries appeared on each side. The faster one (fraction I) showed the same migration velocity as before, the other (fraction II) did not move appreciably during the time of observation. The two fractions were separated and analysed. "Fraction II" was quantitatively quite unimportant. As the ratio between nitrogen and hexosamine was the same in both fractions, the slower boundaries are believed to have been anomalous (δ - and ϵ -effect).¹ Such anomalies are liable to occur in electrophoresis experiments with rapidly migrating substances. This opinion is supported by the fact, that the "schlieren" bands of these two boundaries differed markedly, that on the cathode side being much weaker and more diffuse than that on the anode side.

Fraction I gave a negative biuret reaction. It contained:

Hexosamine	0.412 mg./ml.
Nitrogen	$\left\{ \begin{array}{l} 0.0477 \\ 0.0467 \end{array} \right\}$ 0.0472 mg./ml.

Calculated from the hexosamine content the solution should contain 0.914 mg. hyaluronic acid and 0.032 mg. nitrogen per

¹ See for example TISELIUS and KABAT (1939) and LONGSWORTH and Mc INNES (1939).

ml. A small nitrogen excess is thus found. Calculated as protein it should, however, amount to only about 10 per cent of the hyaluronic acid present. Obviously then the fastest migrating component of the vitreous body is not glycoprotein but pure or almost pure hyaluronic acid.

In another experiment the hyalomucoid was precipitated in the ordinary way from filtered vitreous bodies, redissolved with the aid of alkali and the solution obtained electrophorized. Two boundaries appeared, the faster (F I) showing the same migration velocity as the fastest component in the vitreous body fluid, the slower (F II) migrating with about the velocity of serum albumin. Both components were separated and analysed. F I contained:

Hexosamine 0.42 mg./ml.

Nitrogen 0.076 mg./ml.

The nitrogen excess was slightly greater than in the foregoing experiment. Whether or not this was due to contaminating protein could not be decided. The biuret reaction of F I was negative. F II contained:

Hexosamine 0.04 mg./ml.

Nitrogen 0.51 mg./ml.

The biuret reaction was positive. This component was thus a protein containing about 1.2 % hexosamine.

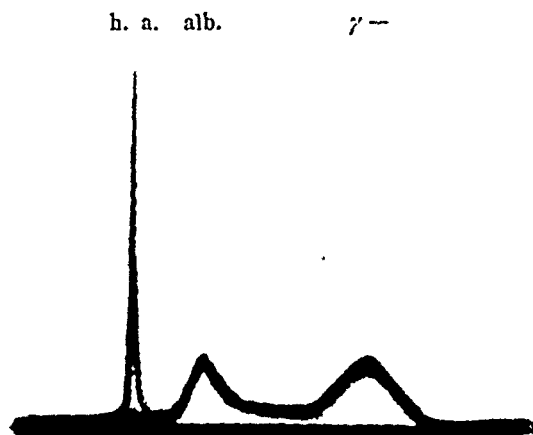


Fig. 1. Concentrated vitreous body fluid. — h. a. = hyaluronic acid.

Fig. 1 shows an electrophoretic diagram of concentrated vitreous body fluid, taken according to the inclined slit method recently

developed by SVENSSON (1939, 1940). It should be noticed that the peak for the hyaluronic acid is completely separated from the protein peak next to it, as this provides evidence against the existence of a loose, dissociable compound between the carbohydrate acid and the protein.

Evidently, then, the hyalomucoid dissolved under neutral conditions is no chemical unit but consists of two apparently uncombined components, viz. hyaluronic acid and a simple protein. The hyalomucoid is an artificial compound, formed on acidification of neutral solutions containing hyaluronic acid and vitreous body protein. In the vitreous body the hyaluronic acid is present, probably exclusively, in the form of salt with inorganic bases and not combined with protein.

2. Experiments on synovial fluid and on synovial mucin. —

Synovial fluids from horses were dialysed overnight against water and precipitated with acetic acid. The mucin obtained was redissolved with the aid of alkali, care being taken that the pH resulting from this procedure did not exceed 8. The mucin was precipitated and redissolved in this way four times. Its characteristic physical properties were not changed during this treatment. The solution finally obtained was dialysed against the buffer used in the electrophoresis. Two boundaries appeared. The faster had a mobility value of $12 \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec.}^{-1} \cdot 10^5$ at pH 8.03. The faster component of the native synovial fluid shows about the same mobility. In a series of electrophoresis experiments the faster component was separated. In all, 28 ml. of solution containing this component were collected, and after reduction to half the volume submitted to a new electrophoresis. This time also two boundaries appeared, the one rapidly migrating, the other stationary. The non-migrating boundaries were to all appearances of anomalous character. The solution of the migrating substance contained:

Hexosamine 0.620 mg./ml.

Nitrogen $\left\{ \begin{array}{l} 0.0672 \\ 0.0735 \end{array} \right\}$ 0.070 mg./ml.

The biuret reaction was negative. The test with sulfosalicylic acid was likewise negative. Calculated from the hexosamine value the solution should contain 1.375 mg. hyaluronic acid and 0.048 mg. nitrogen per ml.

Here also a slight nitrogen excess was found. Calculated as protein it should amount to only 1/10 of the hyaluronic acid present.

In another experiment the slower migrating component of the synovial mucin was isolated electrophoretically and analysed. The analyses showed:

Hexosamine	0.030 mg./ml.
Nitrogen	0.38 mg./ml.

The biuret reaction was positive. This component was thus an ordinary simple protein. — In a third experiment synovial fluid was dialysed against water for 24 hrs. and then precipitated with acetic acid and filtered. The filtrate was analysed for hexosamine and nitrogen. The hexosamine/nitrogen ratio found corresponded to that for a simple protein containing 0.9 % hexosamine.

Fig. 2 shows an electrophoretic diagram of synovial fluid. Also here the peak for the hyaluronic acid is completely separated from the protein peak next to it.

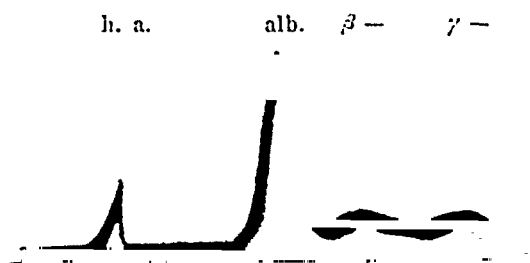


Fig. 2. Synovial fluid. — h. a. = hyaluronic acid.

It is thus clear that the synovial mucin too is an artificial product, formed only on acidifying the synovial fluid with acetic acid. In the synovial fluid the hyaluronic acid appears to be present solely in the form of salts with inorganic bases, and not in combination with proteins.

3. Experiments on submaxillary mucin. —

The submaxillary mucin was prepared according to HAMMARSTEN (1881). It was dissolved with the aid of hydrochloric acid and reprecipitated by dilution with water, this procedure being repeated four times. The mucin finally precipitated was washed free from

HCl and then dissolved in water with the aid of NaOH at a pH not exceeding 8. The solution was thereafter dialysed against the buffer used throughout and submitted to electrophoresis. Submaxillary mucin prepared in this way showed regularly two distinct boundaries, symmetrically on both sides. In Table I the migration velocities of these boundaries at different pH values are given.

Table I.

pH	Mobilities in $\text{cm}^2, \text{volt.}^{-1} \text{ sec.}^{-1} \times 10^5$	
	Faster component	Slower component
6.12	— 7.1	± 0.0
6.80	— 8.3	± 0.0
7.50	— 9.7	— 0.2
8.03	— 10.7	— 0.6

The velocity of the faster component (F I) is somewhat greater than that of serum albumin under the same conditions but not so great as that of hyaluronic acid. The mobility of the slower component (F II) is of the same order as that of the γ -globulin.

In a number of electrophoreses 28 ml. of F I in all were separated. The solution was concentrated to half the volume, dialysed against the usual buffer and once again submitted to electrophoresis. Two components appeared, which were separated and analysed. The slower component was present in such small amounts that the analytical values became uncertain. They are therefore not given here. The solution containing the main, i. e. the faster, component contained:

Hexosamine	$\left\{ \begin{array}{l} 0.512 \\ 0.524 \end{array} \right\}$	0.518 mg./ml.
Nitrogen	$\left\{ \begin{array}{l} 0.514 \\ 0.542 \end{array} \right\}$	0.528 mg./ml.

The biuret reaction was positive.

In another experiment the slower component (F II) of the submaxillary mucin was isolated and analysed. It showed:

Hexosamine	0.032 mg./ml.
Nitrogen	0.099 mg./ml.

Evidently then the submaxillary mucin as prepared in this work is electrophoretically not quite uniform. In approximately neutral solutions the main component is a glycoprotein containing about 27 % polysaccharide acid (mol. weight 381). A minor part, approximately 10 % of the whole, is constituted of a protein with a hexosamine content of about 5 %. As this protein shows a mobility of about the same order as the γ -globulin, it is unlikely that it contains an acid polysaccharide.¹ It probably ought to be regarded as a contaminant.

In one experiment submaxillary glands were repeatedly extracted with water. The extracts still coloured by haemoglobin were rejected. The first practically colourless extract was directly submitted to electrophoresis. Two components appeared, showing identical mobility values with those of the submaxillary mucin. The faster one was separated and gave the characteristic colour reactions for submaxillary mucin. It is thus clear that not even the aqueous extract of submaxillary gland from which the mucin is prepared contains any unbound polysaccharide.

4. Experiments on cartilage extracts. —

100 g. of cartilage (nasal septums from cattle) were extracted at room temperature for two days with distilled water. The extract was centrifuged and filtered. The filtrate was reduced to half the volume at room temperature and dialysed against the usual buffer. The solution was then electrophorized in the larger apparatus of TISELIUS (1938). Two components appeared. Both were isolated and analysed. In a special experiment their electrophoretic mobility was determined at pH 8 and found to be 17.0 and 7.9 $\text{cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1} \times 10^5$ respectively. To judge from this result the faster component was in all probability not a protein. The chemical analyses of its solution gave:

Hexosamine	0.208 mg./ml.
Nitrogen	0.077 mg./ml.

The nitrogen excess is somewhat greater than for the hyaluronic acid in the experiments with vitreous body and synovial fluid. Calculated as protein it should amount to about $\frac{1}{3}$ of the total substance (the non-protein part taken as chondroitin sulphuric

¹ The possibility cannot be excluded that by the separation of the slower component some contamination with the faster one may have occurred. This may account for the relatively great carbohydrate content of the former.

acid). However, the solution of the faster component showed a negative biuret test and a negative sulfosalicylic acid reaction. It is therefore believed that this component was almost pure chondroitin sulphuric acid.

The slower component was a protein containing only 0.02 % hexosamine.

With water alone only a minor part of the chondroitin sulphuric acid in the cartilage can be extracted. Clearly then, the above results support the old view of MÖRNER (1889) that the chondroitin sulphuric acid in the cartilage is partly present as alkali salt.

Discussion.

Two typical glycoproteins, the synovial mucin and the hyalomucoid have proved to be purely artificial products. In the synovial fluids as well as in the vitreous body the hyaluronic acid is, to all appearances, present exclusively in the form of salts with the coexisting inorganic bases and not in combination with the proteins. It is not likely that the synovial mucin and the hyalomucoid are unique among the glycoproteins in being artificial products. However, the outcome of the electrophoretic experiments on the submaxillary mucin should remind us of the danger in generalising from a few cases. The submaxillary mucin seems to exist also in neutral solution as a definite compound between protein and carbohydrate. It is very unlikely that the carbohydrate and the protein should have migrated with the same velocity at the different pH values if they had not been in some way combined with each other. It remains to find out the nature of this combination.

The improved knowledge of the chemistry of the glycoproteins gives, in my opinion, no reason for using the terms "mucin" and "mucoid" in any other sense than that in which they have been used in earlier years. "Mucin" should still signify animal *glycoproteins* which occur in or may be prepared from mucus, mucous fluids or mucous tissues. "Mucoid" should signify glycoproteins occurring in or derived from other animal materials. It must be remembered nevertheless that certain glycoproteins are purely artificial products and that the peculiar mucinous character of various materials of animal origin may, in some instances, be due not to a glycoprotein but to a polysaccharide acid alone.

It would, at present, appear perhaps somewhat premature

to attempt a chemical classification of the glycoproteins, nevertheless, it is believed that the classification given below may, as a preliminary one, be of some value.

Glycoproteins.

I. Glycoproteins containing a neutral polysaccharide (*Neutro-glycoproteins*). — Ovo- and sero-mucoid belong to this group.

II. Glycoproteins containing an acid polysaccharide¹ (*Acid-glycoproteins*). This group includes the following sub-groups:

1. *Chondroproteins*, containing chondroitin sulphuric acid. This group includes the chondromucoid and probably all mucoids of the connective tissues.

2. *Hyaloproteins*, containing hyaluronic acid. Only artificial hyaloproteins are as yet known.

3. *Sialoproteins*, containing the acid polysaccharide present in the submaxillary mucin.

4. The term *mucoproteins* should be reserved for eventual compounds between mucic acid sulphuric acid and protein.

Summary.

A short review is given of the results of recent investigations on the chemistry of the glycoproteins and especially of the present state of the question concerning the relations between carbohydrate and protein in these substances.

Hyalomucoid, synovial mucin and submaxillary mucin were investigated with the aid of electrophoresis. In approximately neutral solutions the hyalomucoid and the synovial mucin proved each to contain two electrophoretically well-defined components. In both cases the faster migrating component was found to consist of a pure polysaccharide acid (hyaluronic acid). The slower components were simple proteins. In native synovial fluid and native vitreous body fluid the hyaluronic acid in all probability is in no way combined with protein but exists solely in the form of salts of the inorganic bases present. The glycoproteins precipitated from neutral synovial fluid or vitreous body fluid on addition of acetic acid are to be regarded as purely artificial products.

¹ There appears to be a definite need of a common name for the acid polysaccharides occurring in animal tissues. With regard to the close relations of most of these acids to mucins and mucoids the name "*mucinic acids*" (Mucinsäuren, acides muciniques) is proposed for these acids.

Neutral solutions of submaxillary mucin were, on electrophoresis, found to contain two distinct components, both proteins. The main component, which was the faster migrating one, amounted to about 90 % of the whole and was a glycoprotein containing 27 % carbohydrate. The other component probably ought to be regarded as a contaminant.

Pure water extracts from cartilage contain chondroitin sulphuric acid exclusively in the free form. This confirms the old opinion of MÖRNER that the chondroitin sulphuric acid in the cartilage is partly present as alkali salt.

A chemical classification of the glycoproteins is suggested.

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A Colorimetric Method for the Determination of Potassium in 0.01—0.1 cc. of Blood Serum.¹

By

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(With 1 figure in the text.)

In determining the potassium content of serum it sometimes proves necessary to use such small quantities of serum as 0.1 c.c. For that purpose a method has been worked out by the author which is applicable to this quantity and also to a still smaller amount, 0.01 c.c. of serum, e. g. in determining simultaneously chlorine ions electrometrically, for which only 0.02 c.c. of undiluted serum is needed.

Of the methods proposed for the quantitative determination of potassium in serum, those are the best ones in which potassium is precipitated as potassium sodium cobaltinitrite and cobalt (SOBEL and KRAMER) or nitrite is determined colorimetrically. The precipitation is preferably made in a buffered solution at pH 5.6, according to KRAMER and TISDALL, at which acidity the serum proteins remain in solution. TISCHER has shown that such minute amounts of potassium as 1 γ can be quantitatively precipitated as cobaltinitrite and the nitrite determined with accuracy colorimetrically after adding Riegler's naphthol reagent.

TAYLOR has worked out a method for 2 c.c. serum. He precipitates K in the deproteinized serum filtrate as cobaltinitrite and determines the nitrite with Ilosvay's reagent (sulfanilic acid and α -naphthylamine). Seeing that this reagent allows a determination of very small amounts of nitrite, it has successfully been applied in this case. The sodiumcobaltinitrite-reagent of KRAMER-

¹ Received for publication 30 March 1940.

TISDALL has been slightly modified. As recommended by JACOBS and HOFFMAN the precipitate was washed with distilled water and 70 per cent alcohol.

Principle.

The potassium is precipitated directly from the serum as $K_2NaCo(NO_2)_6$ at pH 5.6—6, by adding a buffered solution of sodium cobaltinitrite. The cobaltinitrite precipitate is dissolved in alkali. Colour is developed by means of Ilosvay's reagent.

Reagents.

1. A buffered sodium cobaltinitrite solution.

30 Gm. of $Na_3Co(NO_2)_6$ and 10.22 Gm. of NaAc are dissolved in 210 c.c. of H_2O . To this 0.47 c.c. of glacial acetic acid are added. The reagent is kept in an ice-chest. It is to be filtered before use. It can be stored for 1—2 months.

2. Distilled water saturated with octyl-alcohol.

This reagent is prepared by shaking 200 c.c. of water with 2 c.c. of octyl-alcohol and then filtering through hardened filter-paper.

3. 70 vol. per cent alcohol.
4. 2 per cent (0.5 N) NaOH.
5. Ilosvay's reagent.

0.5 Gm. of sulfanilic acid and 0.2 Gm. of α -naphthylamine are dissolved in 320 c.c. of HAc (60 c.c. of glacial acetic acid pro 1,000 c.c. of solution) The reagent is destroyed if heated and it is to be kept in the dark. It must be colourless.

0.1 cc. of serum.

Method.

In a small Pyrex centrifuge tube (6×50 mm) exactly 0.1 c.c. of serum (not hemolysed) is pipetted off. To this 4×0.05 c.c. of $Na_3Co(NO_2)_6$ -reagent are added, shaking thoroughly after each portion of 0.05 c.c. being added. The tubes are left at room-temperature for 2 hours. Then 0.2 c.c. are added of reagent 2. After centrifuging about 20 minutes at 3,000 r. p. m. the liquid is carefully decanted, and the tube is allowed to stand upside down on a filterpaper (5 min.) and dried with filterpaper. The precipitate is washed by thoroughly suspending in another 0.5 c.c. of reagent 2 and then centrifuged (5—10 min.). The liquid is

decanted, and the tube is dried upside down again. Then it is washed twice in the same way with 0.5 c.c. of alcohol (70 %) each time. After drying it the second time, the precipitate is dissolved in 0.5 c.c. of NaOH (2 %) and then the tube is put in a boiling water-bath for 10 minutes. Care is to be taken that no trace of the precipitate remains on the walls of the tube. This is best avoided by stirring with a glassrod. This procedure is the most important one. The solution is poured into a 100 c.c. measuring-flask, and the tube is washed 3 or 4 times with reagent 2. The glass-rod is also washed, and the wash-water is poured into the measuring flask, which is filled to the mark with distilled water. For the determination of nitrite, exactly 5 c.c. hereof is transferred to a 25 c.c. measuring-flask and 5 c.c. of Ilosvay's reagent are added. Also this measuring flask is filled to the mark with distilled water. After 10 minutes the red colour is determined colorimetrically in a Pulfrich-Photometer, with colourfilter S_{53} and 3 cm cuvettes against a solution of 5 c.c. of Ilosvay's reagent diluted to 25 c.c. with distilled water.

Calculation.

If the determination is carefully carried out according to the above method, the K of serum is calculated as follows.

$$\text{mg}\% \text{ K} = 55.0 \cdot E_{53}$$

E_{53} = the extinction-coefficient $\left(-\log \frac{I \text{ post}}{I \text{ ante}} \right)$ for 3 cm's cuvettes with colourfilter S_{53} .

0.01 cc. of serum.

Serum is diluted in the proportion 1 : 10.

Of this 0.1 c.c. is pipetted into a small Pyrex-tube (6 × 50 mm). To this 4 × 0.05 c.c. of $\text{Na}_2\text{Co}(\text{NO}_2)_6$ -reagent are added, shaking it thoroughly after each portion being added. The tube is put into an ice-chest (0° C) and is left there overnight (10—12 hours). Then the operation is carried on according to the method for 0.1 c.c. of serum. After dissolving the precipitate the solution is transferred directly to a 25 c.c. measuring flask. To this 5 c.c. of Ilosvay's reagent are added and the flask is filled to the mark with distilled water. Colorimetric reading as above.

Calculation.

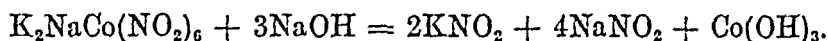
$$\text{mg}\% \text{ K} = 27.50 \cdot E_{53}.$$

Controls.

At the first washing with octyl-alcohol and distilled water, there is no risk of the precipitate being dissolved, because there is still $\text{Na}_3\text{Co}(\text{NO}_2)_6$ present. The octyl-alcohol is used to make the washing easier.

$\text{K}_2\text{NaCo}(\text{NO}_2)_6$ is almost insoluble in 70 per cent alcohol. The wash-water from the 3 rd. washing gives no nitrite-reaction with Ilosvay's reagent; the second, however, gives a noticeable reaction. There is no risk of precipitating $\text{Na}_3\text{Co}(\text{NO}_2)_6$ with 70 per cent alcohol.

When the precipitate is dissolved in NaOH the following reaction takes place.



That nitrite can be determined quantitatively with Ilosvay's reagent according to the method above, is shown in table I, where the formula $c = K_1 \cdot E$ (c = the amount of nitrite expressed in γ . K_1 = constant. E_{53}^{53} = extinction-coefficient) is proved to be valid.

Table I.

γ of NO_2 presents as NaNO_2	Reading of Photometer S_{53}	$K = \frac{c}{E_{53}^{53}}$	Deviation from the average value %
1.60	67.50	9.4	- 3.1
3.20	45.15	9.8	- 4.1
4.80	32.85	9.9	+ 2.1
6.40	22.0	9.7	± 0
8.00	15.40	9.8	+ 1.0
9.60	10.50	9.8	+ 1.0

Average value 9.7.

Thus $\gamma \text{ NO}_2 = 9.7 \cdot E_{53}^{53}$.

The K calculated as $\text{K}_2\text{NaCo}(\text{NO}_2)_6$ will thus be

$$K = 2.75 \cdot E_{53}^{53}.$$

That the precipitate has the formula $\text{K}_2\text{NaCo}(\text{NO}_2)_6$ is shown in table II. Here the K -level is calculated from the nitrite of the precipitate, according to the above formula. As the value obtained in this way, closely corresponds the known value of K , the formula must be correct.

Table II.

K-level in solution	Time for the precipitation	Dissolved precipitate used for the nitrite determination	Colourfilter S_{53}	K-level obtained	Difference %
1.31	12 h. 0° C	1/1	32.5	1.34	+ 2.3
1.81	12 h. 0° C	1/1	33.5	1.31	± 0
1.81	12 h. 0° C	1/1	32.0	1.86	+ 3.8
13.1	2 h. 20° C	1/20	57.0	13.5	+ 3.1
13.1	2 h. 20° C	1/20	58.5	12.8	- 2.3
13.1	2 h. 20° C	1/20	56.5	13.65	+ 4.2

In figure I the amount of potassium precipitated is plotted against the time. It is shown that the concentration of potassium influences upon the time necessary for complete precipitation. From these curves the time necessary for complete precipitation has been selected.

The importance of adding the sodium cobaltinitrite in small portions has been pointed out by KRAMER and TISDALL.

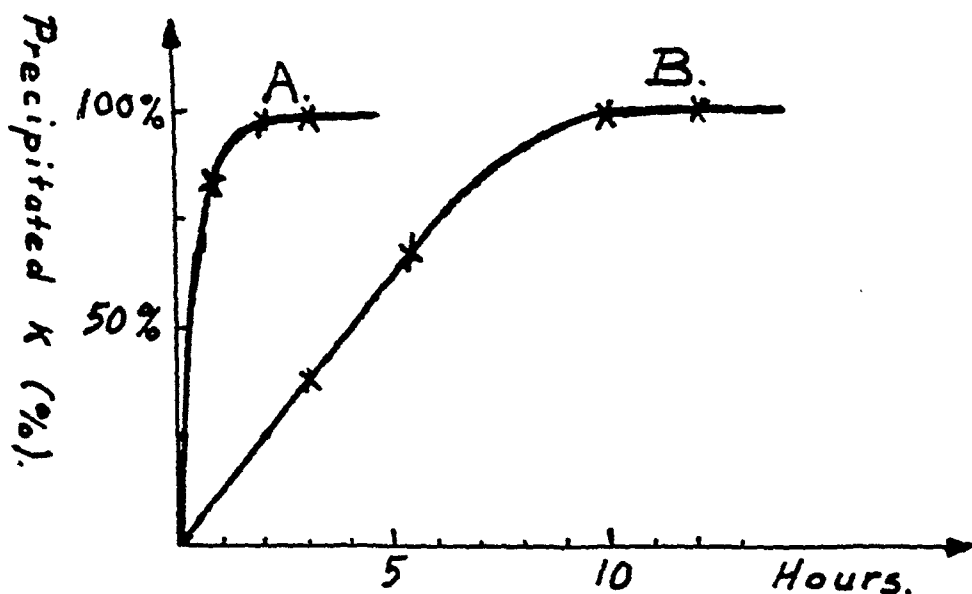


Fig. 1. Curves showing the completeness of the precipitation of different amounts of potassium at different time intervals. The ordinate = percentage of potassium precipitated, the abscissa = the time needed for precipitation. The curve A applies to K-amounts between 13 and 30 γ precipitated at 18–20° C. and the curve B between 1.3 and 3 γ of K precipitated in the cold, 0° C.

In table III determinations have been made with 0.01 and 0.1 c.c. of serum with and without addition of potassium.

Table III.

Quantity of serum c.c.	K-level in serum mg%	Quantity of K added mg%	Total quantity of Kmg%	Obtained quantity of Kmg%	Difference %
0.01	27.0	13.0	40.0	39.1	- 2.25
0.01	19.2	16.0	35.2	34.6	- 1.7
0.01	17.3	16.8	34.1	34.5	+ 1.2
0.1	25.8	12.5	38.3	37.8	- 1.3
0.1	26.5	12.2	38.7	39.5	+ 2.0
0.1	20.4	10.0	30.4	30.2	- 0.7

Table IV shows some values found in normal blood of man and rabbit.

Table IV.

	Quantity of serum c.c.	K-level mg% K
Man	0.01	19.2
	0.1	19.4
	0.01	17.3
	0.1	17.5
	0.01	20.4
	0.1	20.3
Rabbit	0.01	26.9
	0.1	27.0

Summary.

A method has been described for determining the potassium content in 0.01—0.1 c.c. of serum. The accuracy of the method is satisfactory. Added amounts of potassium are quantitatively recovered.

The author's thanks are due to Dr. E. JORPES at who's suggestion this investigation was taken up.

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A New Method for the Determination of Carbon Monoxide in Blood.¹

By

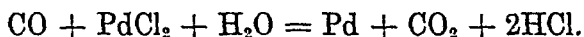
R. WENNESLAND.

(With 4 figures in the text.)

On working with experimental carbon monoxide poisoning the following method for quantitative determination of carbon monoxide in blood was evolved. The method requires no special apparatus, is very easy to perform and the accuracy is great.

Principle.

The method depends upon the ability of carbon monoxide to reduce palladium chloride after the following equation:



This principle has been much employed especially for the detection and determination of carbon monoxide in air. A convenient method for determination of carbon monoxide in blood depending upon the same principle has been elaborated by CHRISTMAN & RANDALL (1933). I have used their method as a starting point. It includes two different steps:

1. The blood gases are extracted by reduced pressure after admixture of acid ferricyanide, and passed over to a reaction chamber containing a known solution of palladium chloride. The connection between extraction chamber and reaction chamber is closed.

2. After the reduction has taken place, excessive palladium chloride is determined colorimetrically.

¹ Received for publication 10. March 1940.

On both steps I thought improvements could be made. At 1: It seemed unnecessary to use reduced pressure for the extraction of carbon monoxide. Regarding the latter, the diffusion rate depends upon the ratio between the partial pressure of the carbon monoxide in the liquid phase and in the gaseous phase. The presence of pure air does not interfere with that ratio. The effect of reduced pressure is merely a mechanical one caused by the bubbling. It seemed also unnecessary to transfer the extracted gases to the reaction chamber and close the connection between extraction chamber and reaction bulb. The gases can wander by their own kinetic power, and the connection between the two

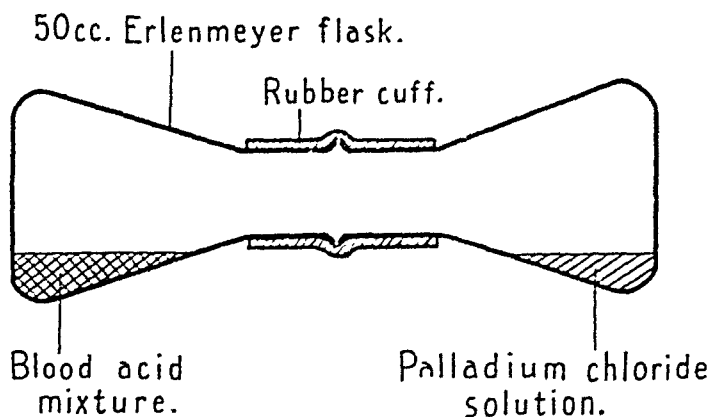


Fig. 1. Analysis apparatus set up with blood acid mixture in the left Erlenmeyer flask, palladium chloride solution in the right.

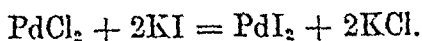
chambers may stand open as long as the blood reagent mixture and the palladium chloride solution do not come in contact with one another.

On this line of argument I made my apparatus, consisting of two Erlenmeyer flasks fastened together at the open ends by means of an airtight rubber cuff (Fig. 1).

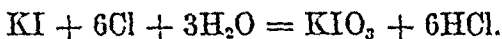
Before they are connected, the blood reagent mixture is placed in one of the flasks, a known solution of palladium chloride in the other. The connected flasks are kept in a horizontal position, carbon monoxide diffuses from the blood through the air to the palladium chloride, where a corresponding quantity of the latter is reduced to metallic palladium.

At 2: As to the determination of the excessive palladium chloride I found the colorimetric method unsatisfactory. In the quantities which I used, I could determine the palladium chloride

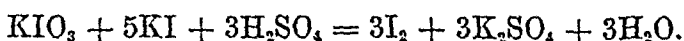
colorimetrically with a standard deviation of ± 0.0175 mg. which represents in carbon monoxide ± 0.22 volume per cent, supposing that 1 cc. of blood has been used. With photometric determination I got only insignificantly better results. I worked out a titrimetric method depending upon the following reaction:



The palladium chloride is precipitated with a known quantity of potassium iodide and the excessive potassium iodide is determined iodometrically after the principle of DUPRÉ-WINKLER: Iodide is oxidised to iodate by means of chlorine water (Von WESZELSKY 1900, WINKLER 1915) or better bromine water (BUGARSKY & HORVATH 1909,) after the following equation:



Superfluous chlorine is removed by boiling, or if bromine has been used, it can also be removed by the addition of phenol in excess (SCHULEK & STASIAK 1928.). From the potassium iodate formed, iodine is released by adding excess of potassium iodide in strong acid solution according to the equation:



With this method I could determine excessive palladium chloride with a standard deviation of ± 0.00137 mg. which represents in carbon monoxide, supposing 1 cc. of blood has been used, ± 0.017 vol. per cent, that is 13 times the precision of the colorimetric method.

Precipitation with potassium iodide for the determination of palladium chloride is an old method, but I cannot find that anyone has applied the principle in the above mentioned manner before. ISHISAKA (1937) has published a method for the determination of carbon monoxide in air based upon the palladium chloride reaction, where "the concentration of PdCl_2 is determined by titrating with KI solution."

Reagents.

10 per cent sulfuric acid for the transformation of carbon monoxide hemoglobin into sulfuric acid hematin. CHRISTMAN & RANDALL used ferricyanide after HALDANE (1897—98) mixed

with lactic acid, to free the carbon monoxide. I found however that during the interval until the reduction of palladium chloride is maximal (3—4 hours), a certain amount of the acid ferricyanide is converted into a volatile substance, probably hydrocyanic acid, which reacts with the palladium chloride and gives higher values for the carbon monoxide than actually present. E. g. a blood sample containing 5.30 vol. per cent of carbon monoxide gave values of 8.48 vol. per cent after 4 hours, 9.05 vol. per cent after 13 hours, when treated with acid ferricyanide.

1/400 N potassium iodide solution containing 1 per cent of aluminium sulfate. — 0.415 grams of potassium iodide p. a. are dissolved in 1 liter of distilled water containing 10 grams of aluminium sulfate. The solution is stored in a dark bottle. The aluminium sulfate serves to precipitate colloid metallic palladium and palladium iodide (CHRISTMAN & RANDALL).

Saturated bromine water produced by shaking pure bromine with distilled water and stored in a little bottle with glass stopper.

1/200 N sodium thiosulfate solution. — 1.241 grams of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ are dissolved in 1 liter distilled and boiled water containing 10 grams of amyl alcohol. With amyl alcohol as a conserving fluid the thiosulfate solution keeps its concentration very well for months.

1/200 N potassium iodate solution for the adjustment of the thiosulfate. — 0.3567 grams of potassium iodate p. a. (MERCK) are dissolved in 2 liters of distilled water and kept in a glass-stoppered bottle in a cool place.

1 per cent starch solution. One gram of soluble starch is dissolved in 100 cc. of boiling water saturated with sodium chloride. I have also added 1 per cent of amyl alcohol for conservation instead of sodium chloride.

2.5 per cent phenol in 2.5 per cent sulfuric acid. A 5 per cent solution of phenol in water is mixed with equal parts of 5 per cent sulfuric acid.

1/100 N palladium chloride solution. — 0.8881 grams of PdCl_2 p. a. (MERCK) are dissolved in 100 cc. of 1/10 N boiling hydrochloric acid. After cooling to room temperature the solution is diluted to 1 liter. Kept in small bottles with glass stoppers the reagent keeps its concentration very well for long periods (Table I). In accurate working however, blank analyses will be made, and it is of minor importance how the palladium chloride solution is stored and how it keeps its concentration.

Table I.

The Durability of the Palladium Chloride Solutions.

Calculated concentration of the various solutions	Age of solutions	Concentration of the solutions determined after present method	
		Stored in clear bottles	Stored in dark bottles
	Days	Normality	Normality
Solution 1: 0.176 gm. of PdCl_2 dissolved in 200 cc. of dilut. HCl $N = 0.00985$	0	0.00976	
	11	0.00977	
	12	0.00977	
Solution 2: The same concentration	0	0.00979	
	3	0.00977	
	5	0.00978	
	9	0.00979	
Solution 3: 0.350 gm. of PdCl_2 dissolved in 200 cc. of dilut. HCl $N = 0.01970$	0	0.01951	0.01951
	12	0.01944	0.01943
	22	0.01949	0.01948
	31	0.01948	0.01949
	61	0.01961	
	63		0.01941
	64	0.01963	0.01955
	70	0.01946	

Apparatus and Equipment.

Handling of the blood: To prevent the clotting of the blood heparin, potassium oxalate or sodium citrate may be used, as experience has shown that none of these substances interferes with the analysis. I consequently add 0.15 per cent of dry neutral potassium oxalate.

The blood may be handled and stored anaerobically after the directions in PETERS & VAN SLYKE (1932). More convenient however is the use of syringes with glass piston (BARCROFT & HALDANE 1902, SCHOLANDER 1938). The blood sample is drawn into an airfree syringe containing the potassium oxalate or another anticoagulant. The syringe is provided with a small rubber tip, which can be closed by a short nail. Syringes where the tip is

placed excentrically, are especially convenient when small air bubbles are to be removed. The sample can be stored about 2 weeks without changing its content of carbon monoxide when kept at a temperature of 3—5° C. When kept at room temperature, the evolution of putrefactive gases will some times interfere with the analysis after 2—3 days. The presence of interfering substances is detected quite easily however (See: Remarks on details).

Apparatus: The form of the apparatus after the principle two chambers in open connection with one another, may naturally

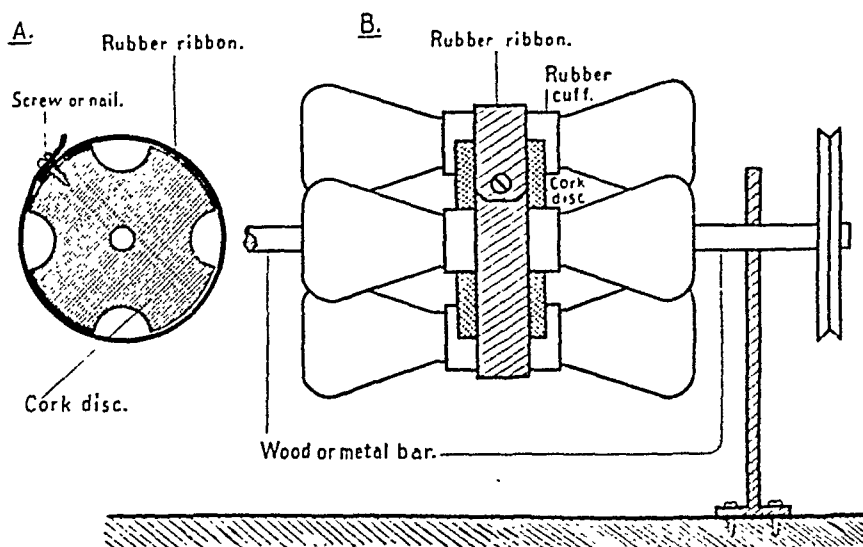


Fig. 2. A. One of the prepared cork discs with rubber ribbon. B. Part of the rotary mechanism with analysis apparatuses placed in one of the cork discs.

be varied in different ways. I have found two 50 cc. Erlenmeyer flasks to be the best, fastened together at the open ends as previously mentioned, by means of a rubber cuff of suitable calibre.

I have found it practical to make a rotary mechanism for the flasks (Fig. 2). A long wood or metal bar penetrates the centre of a series of cork discs placed at a distance of 15 cm. from each other. The corks have a diameter of 7 cm. and a thickness of 3 cm. Four small nicks are cut in the circumference of the corks, where the neck of the flasks can rest firmly. The flasks are kept in place by a 2—3 cm. broad rubber ribbon, which is tied once around the cork with the flasks and fastened on a nail. The bar rests horizontally on two bearings and is rotated by a motor 50—60 times a minute. In series analyses especially the rotary mechan-

ism is practical. By making the bar long enough and using large corks, there is room for a large number of flasks.

Of adjusted pipettes a 1 cc. pipette is needed for the blood, further a 2 cc. a 5 cc. and a 10 cc. one. All pipettes are adjusted with water for delivery after the instructions in PETERS & VAN SLYKE (1932).

For the final titration a 10 cc. burette with 1/100 cc. gradations is used. With a fine glass tip droplets of 0.01—0.02 cc. can be obtained. The burette is adjusted with water.

Procedure.

Both of the flasks must be clean and dry. One of the flasks is prepared with the rubber cuff on its neck and 2 cc. of palladium chloride solution in it. The other flask contains 3 cc. of distilled water. 1 cc. of blood is delivered slowly under the water. The flask is quickly shaken to mix the blood and the water, $\frac{1}{2}$ cc. of 10 per cent sulfuric acid is dropped into the blood water mixture, and as quickly as possible the flasks are connected in a horizontal position without letting any of the blood acid mixture come in contact with the palladium chloride.

The flasks are placed in the rotary mechanism and rotated for 3—4 hours, while the reduction of palladium chloride takes place. Then they are disconnected and the excessive palladium chloride is determined in the following manner: To the flask containing a mixture of metallic palladium and palladium chloride, 10 cc. of the $KI-Al_2(SO_4)_3$ mixture is added, and the flask is shaken vigorously. The palladium iodide and metallic palladium will form a fine dark brown precipitate, which is separated from the solution by filtering through a dry filter into a dry flask. From the filtrate an aliquot portion is taken for the determination of excessive potassium iodide. I have found 5 cc. out of a total fluid quantity of 12 cc. suitable. Into these saturated bromine water is dropped until the solution shows a bright yellow colour. 3—4 drops will generally suffice. The flask is shaken and after 1—2 minutes the excess of bromine is removed and the solution made acid by adding 2 cc. of the phenol sulfuric acid mixture and shaking. One minute or so later a crystal or two of potassium iodide p. a. is added and after shaking, the I_2 formed is titrated with the adjusted 1/200 N sodium thiosulfate solution. The titration must be done slowly. When the fluid is nearly decoloured, $\frac{1}{2}$ cc. of the

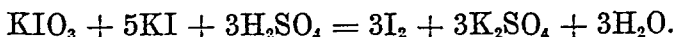
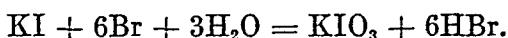
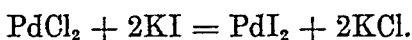
starch solution is added and the titration goes on until the blue colour disappears. With good starch solution the change will occur with $\frac{1}{2}$ —1 droplet, that is 0.01—0.02 cc. of 1/200 N sodium thiosulfate.

For accurate results blank analyses ought to be made to correct for small quantities of carbon monoxide in the laboratory air which is enclosed in the flasks. The blank analyses are made as the blood analyses regarding all reagents except that the blood is omitted.

Modifications in the procedure: When an analysis has to be done only occasionally, it will not be profitable to make a rotary mechanism. The results however will be as good when the flasks are left lying still at room temperature. In that case the reaction time must be 14—18 hours to reach maximum values. The blank analyses may be omitted and be substituted by a titration of 2 cc. of the palladium chloride solution after the present method now and then. The error introduced thereby is generally negligible.

Calculation.

The concentration of the palladium chloride is calculated after the equations:



The formula for the calculation of the concentration of the palladium chloride thus will be:

$$\text{PdCl}_2 = \frac{1}{6} \left(a - \frac{b}{q} \right) X,$$

where a represents the cubic centimeters of thiosulfate required to titrate ad modum DUPRÉ-WINKLER the quantity of KI added to the palladium chloride solution, b the thiosulfate required to titrate excessive KI, when the PdI_2 precipitated has been removed by filtering. X represents the normality of the thiosulfate adjusted with 1/200 N KIO_3 , and q the aliquot portion taken, generally 5/12.

The carbon monoxide is equivalent to the quantity of palladium chloride reduced, which can be expressed as the difference

between original PdCl_2 (or better PdCl_2 of the blank analysis) and excessive PdCl_2 after the reaction with carbon monoxide.

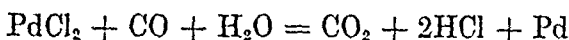
$$\text{CO} = \frac{1}{6} \left(a_1 - \frac{b_1}{q} \right) X - \frac{1}{6} \left(a_2 - \frac{b_2}{q} \right) X.$$

As $a_1 = a_2$ (generally one will add as much KI to the blank as to the blood analysis), the expression is reduced to:

$$\text{CO} = \frac{b_2 - b_1}{6q} X,$$

where b_1 is the cubic centimeters of thiosulfate required to titrate the blank, b_2 is the thiosulfate required for the blood analysis. X and q have the same meaning as previously.

From the equation:



one will see that 1 cc. of N $\text{PdCl}_2 = \frac{22.4}{2}$ cc. of CO at 0°C . and 760 mm. Hg.—To express the carbon monoxide as volume per cent, one will have to multiply the expression above by $11.2 \frac{100}{v}$, where v is the volume of the blood sample. The complete expression for CO in volume per cent of the blood is:

$$\frac{(b_2 - b_1) X 1120}{6 v q}$$

With amylie alcohol added to the thiosulfate X will remain constant, and the expression above can be abbreviated to:

$$\text{CO} = \frac{(b_2 - b_1) f \text{ volume per cent,}}{6 v q}$$

where f is constant $= \frac{1120 X}{6 v q}$ as long as the same concentration of thiosulfate is used, the same volume of blood and the same aliquot portion are taken.

Example: Analysis of 1 cc. of blood has given a terminal excess of KI = 9.45 cc. of 1/205 N sodium thiosulfate.

The blank analysis gave excessive KI = 2.85 cc. of thiosulfate.

The aliquot portion taken was 5/12.

$\text{CO} = (9.45 - 2.85) f$ vol. per cent, where

$$f = \frac{12 \times 1120}{5 \times 6 \times 205} = 2.185.$$

$\text{CO} = 6.60 \times 2.185 = 14.42$ vol. per cent in the blood. (For correction see below under: Results.)

Remarks on Details.

The dilution of the blood with water before addition of sulfuric acid is important. If not well mixed with water, the blood some times will form a clot when sulfuric acid is added, and the analysis can be spoiled. Especially when the flasks are left lying still, the blood acid mixture often will form a gel. This does not however interfere with the analysis. Sufficient acid must be used to convert all hemoglobin into sulfuric acid hematin. $\frac{1}{4}$ — $\frac{1}{2}$ cc. of 10 per cent H_2SO_4 will be abundant for 1 cc. of blood. If all the hemoglobin is not converted into sulfuric acid hematin, the mixture will keep a red tinge, different from the dirty brown colour of the acid hematin. The reaction goes on in the same way even if sulfuric acid is not added to the blood, but much more slowly and the results vary more.

The effect of shaking on the reaction time was examined. The results are presented graphically in fig. 3.

When the flasks are rotated, maximum values are attained after 3—4 hours reaction. After about 6 hours a slight decrease takes place and goes on as long as I have observed it.

When the flasks are lying still, maximum values are attained after 14—18 hours, whereafter a similar decrease takes place. I tried to shorten the reaction time by warming the flasks and the reagents, but with no essential result.

WINKLER (1934) in a publication on a method for determination of carbon monoxide in air, depending upon the palladium chloride reaction, has made exactly the same observation as to the correlation between reaction time and maximum values when the flasks are shaken.

As to the slight decrease in the values for carbon monoxide as the length of reaction time increases, the cause is perhaps as indicated by CHRISTMAN, BLOCK & SCHULTZ (1937) that the oxygen in the air interferes with the reaction of palladium chloride with carbon monoxide.

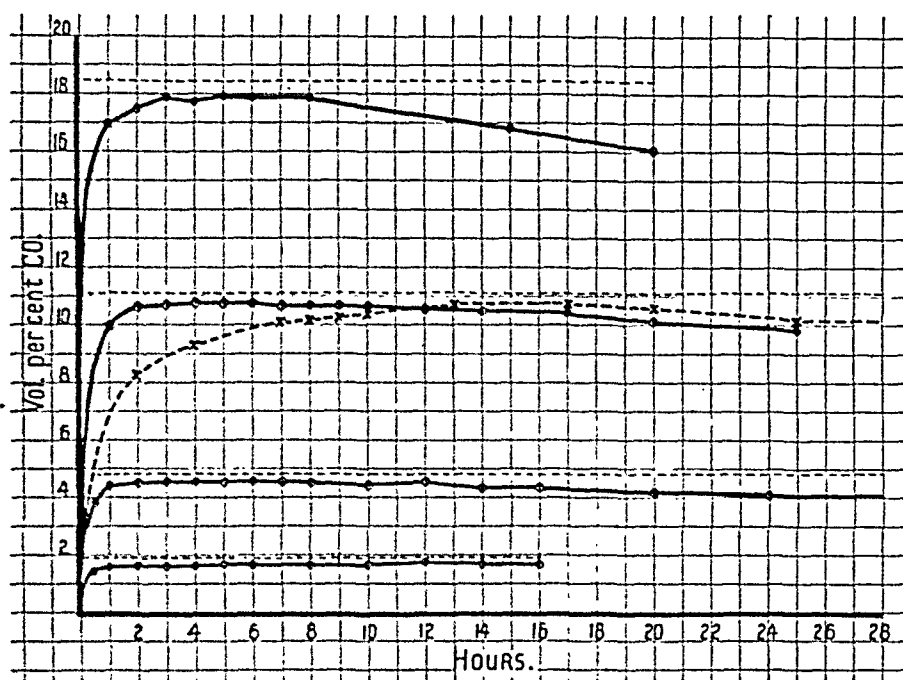


Fig. 3. Analyses of Blood for Carbon Monoxide.

Curves showing the correlation between analysis values and reaction time, when the flasks have been rotated ———, and when they have lain still — — — — —. All curves are empirical. The analysis values are uncorrected. The thin horizontal dotted lines — — — — — represent the corresponding values determined after the method of SENDROY & LIU.

As the figure shows, the maximum values of carbon monoxide after the described method lie about 4 per cent of the values concerned lower than the "real" carbon monoxide content determined after the method of SENDROY & LIU. This error is rather constant for all quantities, as will be seen later, and is naturally explained as a consequence of the interfering process mentioned above.

The palladium chloride solution must not be too acid in view of the later oxidation of excessive potassium iodide with bromine, which according to all authors is appreciably disturbed if the pH is too small. (E. g. REITH (1929) whose suggestions I have followed.) In the filtrate I found the pH about 5.4 before the addition of bromine water. For the oxidation of KI to KIO₃ I have tried both chlorine (WINKLER) and bromine (BUGARSZKY & HORVATH) with equal results. Bromine is preferable because it is more conveniently handled and more easily driven out.

I have also tried other methods for determination of excessive KI, such as direct titration with potassium iodate in strong hydrochloric acid solution, (ANDREWS, after KOLTHOFF 1931,) and the "iodo-cyane method" of LANG (KOLTHOFF 1931). Titration after the principle of DUPRÉ-WINKLER has however given me the best results, probably because the titration error is divided by 6, as will be seen from the reaction equation.

The Erlenmeyer flask containing the palladium chloride solution must be scrupulously cleaned and dried. It can be washed with dilute ammonia and rinsed well with warm water. It is practical always to use the same flasks for the palladium chloride and others for the blood. In the flasks with the blood acid mixture traces of a colloid clot will often adhere to the walls and are difficult to remove. This does not interfere with the analysis, when the same flasks are used for the blood, but some times when I happened to take such a flask for palladium chloride, the solution got brownish, cloudy and gave too low values for the carbon monoxide. Other impurities in the analysis, as when I originally mixed the blood with acid ferricyanide, and sometimes when I examined very decomposed blood, were always detected because they discolored and clouded the palladium chloride solution. I take it as an indication that the reaction is without impurities, when the PdCl_2 solution keeps a bright yellow colour and the metallic palladium after some time floats on the surface like a mirror.

Regarding the precipitation of palladium iodide, BEAMISH & DALE (1938) have stated that at least ten times the calculated amount of potassium iodide may be added without danger of palladium loss. When using reagents of the same concentrations as I do, the ratio $\text{KI}/\text{PdCl}_2 = 10/8$ when no carbon monoxide is present, and $10/1$ when the carbon monoxide content is 19.6 vol. per cent. Up to the latter content the requirements of BEAMISH & DALE are thus fulfilled. If larger quantities are to be examined, a somewhat stronger solution of palladium chloride can be employed, or the blood sample may be reduced to $\frac{1}{2}$ cc. These precautions are advisable also because 2 cc. of $1/100$ N palladium chloride are only capable of oxidizing a theoretical maximum quantity of 22.4 vol. per cent of carbon monoxide in 1 cc. of blood.

For filtering I use white ribbon filter paper, SCHLEICHER & SCHÜLL No. 589². Any filter which does not allow particles of Pd or PdI_2 to pass, is applicable. If traces of Pd or PdI_2 pass the

filter, they will be detected at the final titration with sodium thio-sulfate. The change of colour will be indistinct and the final solution which normally is water clear, will show a red brownish tinge. The same filter can be used for all double analyses.

It is of importance that sufficient bromine is added, but not too much. If too much, it will form with phenol a cloudy grey-yellow precipitate and this interferes with the analysis. After the addition of phenol sulfuric acid mixture the solution ought to remain clear (CLOSS 1931.) In most of my analyses excessive bromine was removed by boiling after REITH's instructions (REITH 1929). Removal with phenol is more convenient and as reliable.

Results.

As control I have used the method of SENDROY & LIU for determination of carbon monoxide in blood (SENDROY & LIU 1930, PETERS & VAN SLYKE 1932) a manometric method which I have modified a little, introducing a syringe absorption pipette instead of the modified Hempel pipette of VAN SLYKE-HILLER. (AUTHOR in press.)

To test the reliability of my own method I produced series of blood samples with various carbon monoxide content. Generally I used defibrinated ox- or calf blood which I obtained from the municipal slaughter-house, some times directly from the animal, in the latter cases prevented from coagulation by means of sodium oxalate. The blood was saturated with carbon monoxide and its content determined after SENDROY & LIU's method.

The saturated blood was mixed with unsaturated, whose quite small content of carbon monoxide had been determined in the same way. Later I found it more convenient and as exact to dilute the saturated blood with distilled water in various proportions. My main series is made in the latter way.

The carbon monoxide content of the various blood water mixtures was determined in two ways:

1. It was calculated from the values of the saturated blood and the known dilution grade ("calculated values").
2. It was determined directly for all dilution steps after the method of SENDROY & LIU ("determined values").

The various dilution steps were: 1/10—2/10—3/10—4/10—5/10—6/10—7/10—8/10—9/10— and undiluted.

Table II.

Analyses of Blood for Carbon Monoxide.

Present method. Reaction time 4 hours. 1 cc. of blood has been used for all analyses. CO content expressed as vol. per cent of blood.

Dilution grade	Number of observations	Vol. per cent of blood α	Standard deviation σ	Coefficient of variation $\frac{\sigma}{\alpha} 100$	Relation between determined values, (table III) and α
$1/10$	9	1.57	0.032	2.0	1.038
$2/10$	25	3.10	0.056	1.8	1.036
$3/10$	29	4.75	0.048	1.0	1.038
$4/10$	53	6.40	0.072	1.1	1.042
$5/10$	17	8.64	0.070	0.8	1.036
$6/10$	20	10.35	0.050	0.5	1.046
$7/10$	23	12.06	0.104	0.9	1.032
$8/10$	24	14.04	0.062	0.4	1.026
$9/10$	22	15.82	0.111	0.7	1.043
$12/10$	20	17.77	0.115	0.6	1.038

Table III.

Analyses of Blood for Carbon Monoxide.

Present method compared with the method of SENDROY & LIU. 1 cc. of blood used for all analyses. CO content expressed as vol. per cent of blood.

Dilution grade	Present method		Sendroy & Lin's method			
	Vol. per cent multiplied by 1.04	Number of observations	»Determined values»		»Calculated values»	
			Vol. per cent	Number of obs	Vol. per cent	Number of obs
$1/10$	1.63	9	1.63	2	1.64	4
$2/10$	3.22	25	3.21	7	3.27	4
$3/10$	4.94	29	4.93	11	4.92	3
$4/10$	6.66	53	6.67	16	6.66	3
$5/10$	8.99	17	8.95	8	8.98	3
$6/10$	10.76	20	10.83	3	10.78	3
$7/10$	12.54	23	12.44	6	12.57	3
$8/10$	14.60	24	14.40	6	14.37	3
$9/10$	16.45	22	16.50	7	16.61	11
$12/10$	18.48	20	18.45	11		

The results are presented in table II and III. As will be seen in table II, the coefficient of variation decreases with increasing carbon monoxide content, until from a content of about 8.5 vol. per cent and higher it reaches an average of about 0.6 per cent of the analysis value.

The coefficient of variation is the standard deviation in per cent of the analysis value. The standard deviation is found after the formula

$$\sigma = \sqrt{\left[\frac{\sum o_i^2}{n} - \left(\frac{\sum o_i}{n} \right)^2 \right] \frac{n}{n-1}}$$

where o_i represents the observations and n the number of the observations.

As previously mentioned the values of carbon monoxide after the present method lie a little lower than the "real" values determined after the method of SENDROY & LIU, and therefore have to be corrected. The relation between the "determined values" and the corresponding values found after the present method was calculated for the various dilution steps. The results are seen in the last column of table II. The relation amounts to an average of 1.0375 and seems to be constant for all quantities of carbon monoxide which are represented in the table, when a reaction time of 4 hours is used.

In a supplementary series of analyses consisting of 113 observations after the present method compared to 58 observations after the method of SENDROY & LIU, where the carbon monoxide content ranged from 3.54 vol. per cent to 18.42, the relation amounted to an average of 1.043.

The values found after the present method therefore have to be corrected by multiplying them by 1.04 to reach the values obtained after SENDROY & LIU. In table III the values of my main series have been corrected and are compared to the so-called "determined values" and "calculated values."

I tried to get a verification of my results in a more direct way by analysing a mixture of air and carbon monoxide under approximately the same circumstances as the blood analyses. Carbon monoxide gas was prepared in the usual way by warming a mixture of anhydrous formic and sulfuric acids. The gas was washed by bubbling it repeatedly through alkaline pyrogallate and analysed in an apparatus resembling the volumetric apparatus of

VAN SLYKE, but of only 10 cc. volume. The apparatus was filled with the gas to the 10 cc. mark, carbon monoxide was absorbed by WINKLER's cuprous chloride solution (prepared after PETERS & VAN SLYKE 1932), and the volume of the rest gases (a little N_2 and water vapour) read on the calibrated upper stem of the apparatus. 10 cc. of the gas analysed were mixed with 1000 cc. of air and 10 cc. of the mixture were introduced into an Erlenmeyer flask of 100 cc. volume containing 2 cc. of the usual palladium chloride solution. The introduction was performed in the following way: When the palladium chloride had been placed into the Erlenmeyer flasks, they were tightly stoppered by rubber

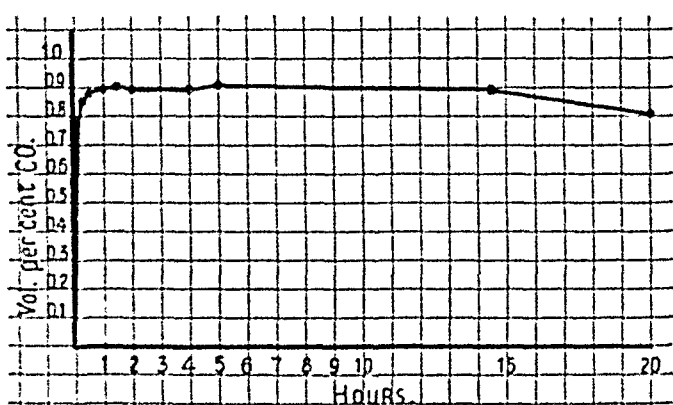


Fig. 4. Analyses of 10 cc. of a Carbon Monoxide — Air Mixture. Empirical curve showing correlation between analysis values and reaction time.

stoppers penetrated by a glass tube with a stop cock. A little of the air in the flasks was sucked out by means of a water pump, sufficient to allow 10 cc. of the carbon monoxide — air mixture to enter. The latter were introduced from the analysis apparatus mentioned above. The tube and its stop cock were sealed with water. A quantity of carbon monoxide corresponding to the content of 1 cc. of blood half-saturated with the gas was in this way introduced into the flasks, which were rotated in the rotary mechanism, and the rest of the analysis was performed as described for the blood analyses. Experiments showed that maximum values were attained after a reaction time of $1\frac{1}{2}$ —2 hours (Fig. 4), indicating that part of the longer reaction time of the blood analyses is due to the slower diffusion rate when the gas has to pass out of the blood reagent mixture through the air, before it enters the palladium chloride solution. The values of the analyses

were corrected for temperature and barometric pressure and compared with the "calculated" concentration of the carbon monoxide in the gas mixture. Blanks were made as for the blood analyses. The results are seen in table IV.

Table IV.

Analyses of 10 cc. of a Carbon Monoxide — Air Mixture.

Present method. Reaction time 1.5—2 hours.

	Calculated CO content. Vol. per cent.	Determined CO content. Vol. per cent.	Relation between calculated and determined values.
Sample 1.	0.956	0.900	1.062
	0.955	0.893	1.070
		0.890	1.074
Sample 2:	0.955	0.912	1.047
New mixture.		0.914	1.045
CO the same.		0.912	1.047
		0.909	1.051
		0.908	1.052
Sample 3:	0.954	0.904	1.055
New mixture.	0.953	0.904	1.055
CO the same.		0.896	1.064
Sample 4:	0.979	0.922	1.062
New CO produced	0.979	0.922	1.062
and mixed.		0.922	1.062
		0.922	1.062

The relation between the "calculated values" and the analysis values are seen to be a little greater than the blood analyses indicate, viz. 1.058 per cent on the average. The mixing of carbon monoxide with air and the transfer of the gas mixture to the analysis apparatus however, include sources of error through small leakages, all of them tending to increase the difference. I am inclined to believe the difference of 4 per cent obtained from the blood analyses to be the most correct one.

It is convenient to include the correction in the factor f (compare the chapter: Calculation), by multiplying it by 1.04. The

Table V.

The Corrected Factor F Determined for 1 cc. of Blood, an Aliquot Portion of $\frac{5}{12}$ and Values of y from 4.90 cc. to 5.20 cc.

(y = the number of cc. of sodium thiosulfate solution required to titrate 5 cc. of 1/200 N standard potassium iodate solution.)

y found cc.	The corrected factor F.	y found cc.	The corrected factor F.
4.90	2.377	5.06	2.302
4.91	2.372	5.07	2.297
4.92	2.367	5.08	2.293
4.93	2.363	5.09	2.288
4.94	2.358	5.10	2.284
4.95	2.353	5.11	2.279
4.96	2.348	5.12	2.275
4.97	2.344	5.13	2.271
4.98	2.339	5.14	2.266
4.99	2.334	5.15	2.262
5.00	2.330	5.16	2.257
5.01	2.325	5.17	2.253
5.02	2.320	5.18	2.249
5.03	2.316	5.19	2.244
5.04	2.311	5.20	2.240
5.05	2.307		

corrected factor is called F. In table V I have calculated F for different concentrations of sodium thiosulfate solutions of about 1/200 N, using the formula:

$$F = 1.04 f = 1.04 \frac{1120 X}{6 v q}.$$

Supposing that 1 cc. of blood has been taken ($v = 1$.) and an aliquot portion of $\frac{5}{12}$ ($q = \frac{5}{12}$.) the concentration of the sodium thiosulfate (X) remains the only variable upon which the factor depends. X is found by titrating 5 cc. of a 1/200 N standard solution of potassium iodate. Calling the number of cubic centimeters of sodium thiosulfate solution required in this titration y, X is found from the equation: $X = \frac{5}{200 y}$. It is however most practical to substitute X by y in the table, so that the factor F can be read

directly when 5 cc. of the potassium iodate have been titrated. The carbon monoxide content is then found from the formula:

$$\text{CO} = (b_2 - b_1) F.$$

If concentrations of sodium thiosulfate are employed which lie outside the quantities of the table, blood samples other than 1 cc. and aliquot portions different from 5/12 are used, F must be determined from the formula above.

Table VI.

Analyses of Blood with Small Contents of Carbon Monoxide.

	Present method.				» Calculated values.» Vol. per cent.
	Cc. analysed	Vol. per cent mult. by 1.04.	Standard dev.	Number of obs.	
Sample 1.	2	0.184	0.019	9	} 0.206
Dilut. 1/100.	5	0.198	0.015	14	
Sample 2.	2	0.093	0.030	12	} 0.103
Dilut. 1/200.	5	0.094	0.015	14	
Sample 3.	1	0.198	0.016	8	} 0.194
Dilut. 1/100.	2	0.193	0.009	10	
Sample 4.	1	0.119	0.022	7	} 0.097
Dilut. 1/200.	2	0.114	0.013	10	

In samples 1 and 2 the CO saturated blood was diluted with water, and the reaction time varied from 18 to 40 hours. In samples 3 and 4 the saturated blood was diluted with fresh blood and the reaction time was constant: 3 hours.

I have finally analysed blood with a very small carbon monoxide content (Table VI). The samples were prepared as described for my main series by diluting CO saturated blood after having analysed it. Samples 1 and 2 were diluted 1/100 resp. 1/200 with distilled water and blanks were made as usual. The reaction time for the analyses of both of them varied much, from 18 to 40 hours, because they were examined before I had detected the correlation between time and analysis value. The long reaction time employed is probably the reason that the usual correction of 1.04 is too small for samples 1 and 2.

Samples 3 and 4 were analysed after I had prepared my main series and developed the technique. Though preliminary experiments had shown that it was indifferent for the exactness of my method whether blood diluted with water or mixed with CO-free blood was examined, I thought that it might perhaps play a part at very small CO contents. Possibly the larger content of colloids in a sample mixed with blood could retain minute quantities of carbon monoxide, which generally had no influence on the precision and accuracy of the method but might be detectable in analyses of very small values. I therefore mixed the saturated blood in samples 3 and 4 with fresh blood, and used corresponding quantities of the latter for blanks to exclude errors through the presence of minute quantities of carbon monoxide in it. A constant reaction time of 3 hours was used. In all analyses in table VI the mean values of the present method are compared to the "calculated values" only, determined by dividing the mean value of 3 analyses of the saturated blood by 100 and 200 respectively.

As will be seen, the precision of the analyses of samples 3 and 4 is excellent. The analyses of samples 1 and 2 are not so precise, but as mentioned above, they were performed before the technique was fully worked out. E. g. the variations in the reaction time used will tend to give a greater scatter of the results. At the small values employed in the last experiments the precision of the blanks will be only insignificantly better than that of the analyses. Increasing the number of the analyses will therefore have no influence on the accuracy of the determination, unless the number of the blanks is increased correspondingly. The analyses of samples 3 and 4 are based upon the mean value of 7 blanks. The results indicate that the method is well fitted for micro-analyses.

If one is only interested in the detection of carbon monoxide in the blood, the present method is a very simple one for the purpose. The long reaction time can be spared, as it is of no interest for the detection that maximum reduction takes place. The titration is omitted and blanks are generally unnecessary. The two Erlenmeyer flasks are prepared as previously described with blood sulfuric acid mixture in one, palladium chloride solution in the other. With blood samples of 1 cc. the smallest quantities of carbon monoxide which are distinctly observable are about 0.5 vol. per cent. The reduction was clearly seen after 20—25 minutes. 2 vol. per cent of CO gave distinct reduction after 10—12 minutes and with 5 vol. per cent and larger it took about 5 minutes to get

a distinct mirror of metallic palladium on the surface of the solution. The flasks were rotated by rolling them on a table. If they were left lying still, distinct reduction took place only 2—3 minutes later. In a few cases, when the flasks were a little greasy on the inside so that the palladium chloride solution did not moisten the walls, no metallic palladium was precipitated. Titration in the usual way showed that the solution contained reduced palladium corresponding to a CO content of 2.70 vol. per cent. Controls showed that it made no difference for the titration whether the palladium was precipitated at once or kept in solution until it was flocculated by the aluminium sulfate in the potassium iodide solution. Values of carbon monoxide from 0.5 to 6—7 vol. per cent can be roughly estimated with practice from the solidity of the palladium mirror. From about 7 vol. per cent and larger it was difficult to see any difference. The quantitative determination however is so simple and convenient that it is preferable in most cases.

Summary.

A new method for the determination of carbon monoxide in blood is described. The blood is brought into an Erlenmeyer flask, mixed with water and its carbon monoxide hemoglobin is converted into acid hematin by addition of sulfuric acid. The carbon monoxide liberated passes into another Erlenmeyer flask, which has been connected with the first one by an airtight rubber cuff and contains a known solution of palladium chloride. A corresponding quantity of the latter is reduced to metallic palladium. Excessive palladium chloride is precipitated by a known quantity of potassium iodide, and the excess of the latter is titrated after the principle of DUPRÉ-WINKLER. The method is well fitted for micro-analysis.

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A Parapharyngeal Method of Hypophysectomy in Rabbits.¹

By

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The observation of hypophysectomized animals is essential for the study of the functions of the anterior lobe of the pituitary body. For this reason special interest has been paid to the method of extirpating the hypophysis. On account of the differences in the anatomy of various animals it has been found necessary to try out a particular technic for each species. Also in such cases where the same route is chosen for exposing and removing the hypophysis, the operation procedure has to be adapted to the animal species concerned. For the rat in particular an excellent method has resulted from the experiments of SMITH (1930), COLLIP, SELYE and THOMPSON (1933) and others. This method, in which the hypophysis is removed by the parapharyngeal route, is comparatively easy to learn. It is true that some have been successful in removing rabbits' hypophyses by buccal (WHITE, 1933) or orbital (FIROR, 1933) approach, but the fact that others (Newton, 1939, COPE and DONALDSON, 1939) have found it necessary to use various modifications shows clearly enough the difficulties of successfully applying these methods.

In an article of 1936 we gave a brief report of a parapharyngeal method of operation for rabbits, agreeing in principle with the method for rats.

The advantage of this method is that it enables a good survey of the operation field. In addition, there is slight or no bleeding, and no risk of infection from the pharynx as there always is with

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the buccal method. On histological examination of the contents of the sella turcica and the hypophysis stalk attached no anterior tissue could be found.

We have performed about 300 operations in conjunction with different studies on the changes of the sexual organs (WESTMAN and JACOBSON 1936, 1937) exhibited by rabbits after hypophysectomy, and in all of them we have used the parapharyngeal technic. Since we have been convinced of its many advantages, we shall now give a more detailed description of the operative procedure. The terms used are similar to those in BENSLEY's «Practical Anatomy of the Rabbit».

The operation is performed on a table large enough to support both elbows. The operation field is best illuminated by a head lamp.

The animal is stretched out on its back and fastened. Its head is fixed with a band hooked around the incisors of the upper jaw and another band laid through the mouth across the upper jaw (fig. 1). After cutting the hair, the anterior part of the neck is washed with alcohol. The instruments and the hands of the operator are washed, but it is not necessary to work under sterile conditions.

Anesthetic. It is advisable to use an injectable anesthetic, at least for a basal narcosis. When choosing the narcotic and dosage, it should be remembered that the narcosis should not last more than two to three hours. We find a combination of morphine and urethane suitable. The animal is injected subcutaneously with 0.005 Gm. of morphine hydrochloride per kilogram of body weight, and half an hour later 0.5 Gm. of urethane (2 cc. of a 25 per cent solution) per kg. of body weight are injected intramuscularly, distributed in two places. Pernocton (sodium. sec. butyl- β -bromallyl-barbiturat) has also been used in our operations. (0.3 cc. of a 10 per cent solution per kg. body weight intravenously.) If necessary ether can be administered during the course of the operation.

Operative technic. The skin and superficial fasciae are divided by a median incision from the jaw to the sternum. A tracheotomy is performed below the thyroid gland by a small transverse incision, and an angular glass tube is inserted into it (fig. 1). The intratracheal part of the tube is about 2.5 mm in diameter. The left submaxillary gland is lifted up and held to one side by a hook or a Péan forceps. This gives clear exposure of the digastricus muscle,

the greater cornu of the hyoid, the hypoglossal nerve and the stylohyoideus major muscle (fig. 1.). If palpation is done at the place shown with the lower arrow in fig. 1, that is, at the origin of the stylohyoideus major muscle, the pointed jugular process of



Fig. 1. The appearance of the operative field after incision of the skin and tracheotomy.

the occipital bone can be felt. Knowing the features of the cranial base (fig. 5), it is then easy to find the mastoid process and from there the site of the basisphenoid. Two angular elevators, about 5 mm. broad, are then inserted bluntly at the place indicated by the upper arrow in fig. 1, which is situated lateral to the hypoglossal nerve and the greater cornu of the hyoid, medial to the digastricus and caudal to the mylohyoideus. All vessels which



Fig. 2. The field of operation after exposure of the longus capitis muscles. A glass hook keeps the esophagus and trachea to one side. The point of the arrow lies against the median vertebral vein which is compressed by the elevator.



Fig. 3. The basisphenoid is exposed. The point of the arrow lies against the cavernous foramen.

appear during this manoeuvre are carefully pushed laterally and dissection is continued in the medial and cranial direction until the two longus capitis muscles and the interlying median vertebral vein come into view. With the help of a hook to keep the esophagus and trachea to one side and an elevator inserted at right angles to the longitudinal axis of the longus capitis muscles, the two muscles and the median vertebral vein are exposed (fig. 2). It is convenient to use an angular glass hook, turned up at one end and connected with a rubber tube which can be fastened to the operating table. The elevator must be of such a size and shape that it lies well against the basisphenoid, so that the median vertebral vein can

be compressed against the bone with moderate pressure. For this it is necessary that the elevator be held steadily, preferably with the left hand keeping the elbow on the operating table. This makes it possible to tear away the vein and detach the muscles from the bone without any bleeding. The bone is exposed, as shown in fig. 3,



Fig. 4. The basisphenoid has been bored through and the hypophysis is exposed.

to such an extent that the entire basisphenoid and the transverse spheno-occipital synchondrosis are brought into distinct view. The synchondrosis is generally easy to see, especially in small animals, because of its bluish colour, and it is always possible to find it by palpation (fig. 5). If one follows the bony groove running cranialward in the middle of the basisphenoid, one arrives at the cavernous foramen (fig. 5), marked by the arrow in fig. 3, which is relatively large in adult animals and which leads into the sphe-

noid sinus. Since there is a copious flow of blood from this sinus, it is stopped up before the basisphenoid is bored by means of small bone wax plugs inserted carefully into the foramen. The bone wax should be of such a consistency that the little rods, about one centimeter long and one millimeter wide, are made sufficiently pliable by the warmth of the operator's hand, and yet it should

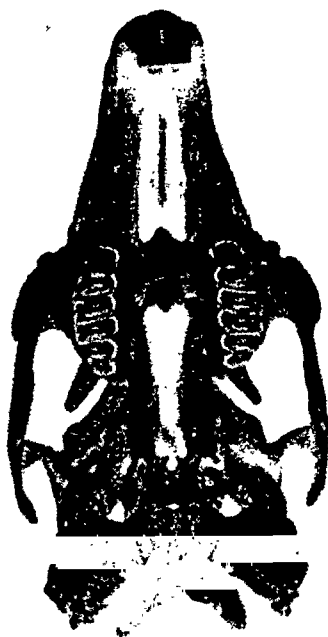


Fig. 5. The cranial base of the rabbit, seen from below.

be so hard that it does not stick to the pincers used to insert it. It is best to use a mixture of two-thirds paraffin with a melting point of 55 C. and one-third cera alba. The rods can be made softer or harder as required by using paraffin with another melting point. When operating upon young animals, it is often impossible to insert these wax plugs, nor is it necessary since the bleeding generally stops of itself as the boring is continued and the spongiosa removed.

The tamponing ought to be finished by the time no more blood oozes from the cavernous foramen. The basisphenoid is bored through with a dental drill placed against the basisphenoid a few millimeters cranial to the sphenooccipital synchondrosis and held absolutely perpendicular. Fig. 6 shows the position and direction of the drill in relation to the skull after the sella turcica has been opened. The opened sella is also seen in fig. 4 where the hypophysis is shown exposed.



Fig. 6. The position of the drill when the sella turcica is opened.

When the bone has been drilled through, the hypophysis is seen clearly under the dura. The dura is slit wide open with a cataract knife and the hypophysis sucked out under visual control through a glass cannula connected with a water vacuum pump. The bore hole is cautiously filled up with bone wax. The glass cannula is removed from the trachea. It is checked that the respiratory passages are not obstructed by blood or mucus (preferably by means of a small suction tube slightly inserted into the trachea). The wound is then closed with skin sutures only.

The operation takes twenty to thirty minutes and can be performed without any assistance. During dissection down to the skull base bleeding ought not to occur and the tamponing being successful very little bleeding will take place during the whole course of the operation.

If the animal does not begin to eat spontaneously the day after the operation, it is good to inject about 30 cc. of a ten per cent solution of glucose subcutaneously several times a day.

Once the operator has mastered the technic, he will have but few deaths and, if one has healthy animals and tends them carefully after the operation, the mortality can be brought down almost to zero.

Summary.

A technic of hypophysectomy in rabbits is described. The parapharyngeal route is taken and the operation done according to the principles of hypophysectomy in rats, but modified to suit the anatomy of the rabbit. The method has great advantages. It enables direct inspection of the field of operation. The operative risk is slight. The mortality is extremely low.

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Sulfapyridine in Secretin Stimulated Pancreatic Juice and Bile of Cats.¹

By

A. TAYLOR² and G. ÅGREN.

For some time past, part of the interest of this laboratory has been devoted to work with secretin and its physiological and therapeutical values (ÅGREN 1939; HAMMARSTEN 1939). In this article we present the results of experiments combining the use of secretin and sulfapyridine in connection with the presence of the latter in the pancreatic juice and bile. It would seem that the pancreas and liver in excreting this drug into the duodenum with the pancreatic juice and bile display the same type of activity as has been noted with the excretion of injected urea and uric acid (ÅGREN 1935).

Amounts of sulfapyridine or sulfanilamide have already been found in pancreatic juice and bile. MARSHALL c. s. (1937 a) commented shortly on the presence of the drug in pancreatic juice and saliva of the dog, CARRYER and IVY (1939) have found it in the secretions of all digestive glands of the dog, and BETTMAN and SPIER (1939) observed it in human bile. Our work confirms these results and approaches the problem from a different angle — a combination treatment of secretin and sulfapyridine.

We have also interested ourselves in the change of the drug from free to bound form within the system. In this connection STEWART (1939), ROURKE and ALLEN in working with rabbits

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conducted *in vivo* experiments to show that the acetylation of sulfanilamide takes place in the liver, and KLEIN and HARRIS (1938) previously found the same thing in *in vitro* experiments with rabbit liver slices.

All work has been performed on cats, in whom the pancreatic juice and bile were collected separately to determine the drug content of each. One impelling reason for using cats rather than other animals was that they are similar to man in the change of the drug inside the body from free to bound form (MARSHALL c. s. 1937 a).

Methods. The method used in determining the amount of sulfapyridine in blood, pancreatic juice and bile is essentially that described by MARSHALL c. s. (1937 b and 1938). The readings were made in a Pulfrich Photometer with an S50 Jena filter and compared with standard curves. For total drug analysis in all fluids, alkaline hydrolysis was used being slightly more effective than acid after the method of BAINES and WIEN (1939).

All injections of sulfapyridine and secretin as well as the removal of blood were made in the femoral veins.

Since the pancreatic duct in the cat does not allow of cannulation, pancreatic juice was collected on tightly-rolled filter-paper wads immediately on its secretion into the duodenum (ÅGREN 1935). These were boiled out with water four times to a total volume of approximately 75 cc, and filtered. The solution was then evaporated *in vacuo* to about 5 cc, or the approximate volume of the pancreatic juice collected. This was washed out and diluted to 10 cc. Then to determine the volume of pancreatic juice, a one cc portion of this solution was made acid with 10 cc N/100 HCl and titrated against N/100 NaOH. Since the bicarbonate concentration of evenly flowing pancreatic juice may be taken as N/10, the total volume can be calculated.

Bile was collected by cannulation of the Ductus choledochus after previous ligation of the Ductus cysticus to ensure the flow of hepatic bile only. To precipitate the bile pigments, which interfere with color readings, the following method was used. Approximately 0.3 cc bile were diluted to 3 cc with distilled water and made slightly alkaline with 0.5 cc N/10 NaOH. Then 0.5 cc of a 4.5 % solution of ZnSO_4 were added to precipitate the proteins and with them some of the closely associated pigments. 0.6 cc N/1 NaCO_3 were then added, immediately followed by 0.5 cc N/1 BaCl_2 . This fresh precipitate of BaCO_3 brings down the remaining bile

pigments, and when filtered after 10 minutes a clear, colorless solution is obtained. There is no adsorption of the drug during this procedure.

The cats, under urethane narcosis, were given sulfapyridine intravenously (about 100 mg per kilogram body weight) in 4 portions at 5—10 minutes intervals. Individual cat differences were such that the minimum time for these injections varied, but less than 5 minutes tended to produce vomiting. The sulfapyridine injected in the first cats was in a specially prepared form (15—25 % solutions) intended for intravenous injection. It was obtained from Pharmacia, Stockholm, under the name Septiglucon. Unfortunately, analysis showed that only 5 % of the sulfapyridine in solution was in the free form, a concentration too low to permit observation of change in form from free to bound. However, a 20 mg. per cc solution of sulfapyridine, 45 % of which was unbound, was obtained by dissolving 1 gram of the recrystallized, powdered drug in 50 cc of hot 12 % glucose solution. This was more than adequate to determine the difference between pancreas and liver activity in changing the form of the drug.

In all cats, the first injection of secretin (Pancreatest, from Astra, Södertälje, Sweden) was made between 10 and 20 minutes after the last sulfapyridine injection. The amounts of secretin injected (approximately 0.3 mg per kilogram body weight) were calculated to produce maximal secretion, and continued doses during the course of the experiment gave a continuously even flow juice. Immediately before the first secretin treatment, 1.5 cc of blood were taken from the femoral vein, so that the standard could be had against which the pancreatic juice and bile could be compared. Another blood sample was taken at the end of the experiment, and in some cases one or more were also taken during the course of the experiment.

In Table 1 may be seen the general outlook of the experiments. In all cases, "experiment" refers to the period over which secretin was injected. The time between the last sulfapyridine injection and the first one of secretin is not the same in all cases, although the variance is no greater than ten minutes.

Table 2 compares rate of flow of pancreatic juice and bile with the concentration of the drug. In Cats III and IV, the juices were fractionated (represented by a, b, c, d) — III in half hour periods, and IV in fifteen minute periods. The values were calculated for periods of 30 minutes.

Table 1.

Concentrations of Sulfapyridine in Blood, Pancreatic Juice and Bile after Secretin.

	Cat I	II	III	IV	V	VI
Amt. drug given per kg body wt . . .	80 mg	96.5 mg	112 mg	110 mg	92 mg	81.5 mg
Blood conc. <i>before</i> exp	13.8 mg%	9.55 mg%		10.6 mg%	15.8 mg%	12.0 mg%
Blood conc. <i>after</i> exp	5.8 ,	3.8 ,	2.7 mg%	2.5 ,	7.8 ,	8.8 ,
Conc'n in Pan. Juice	5.11 ,	5.5 ,	5.0 ,	1.9 ,	7.5 ,	12.5 ,
Conc'n in Bile . . .		19.2 ,	359.8 ,	102.5 ,	34.1 ,	59.6 ,
Length of Experiment	60 min.	60 min.	90 min.	60 min.	45 min.	45 min.

Table 2.

Concentrations of Sulfapyridine in Relation to Rate of Flow of Pancreatic Juice and Bile.

	Pancreatic Juice		Blood Conc'n of Sulfap. mg/100 cc	Bile	
	Rate of Flow cc/30 min.	Conc. of Sulfap. mg/100 cc		Rate of Flow cc/30 min.	Conc. of Sulfap. mg/100 cc
Cat I	2.2	5.11	5.8		
Cat II	2.5	5.5	5.8		
Cat III	a) 2.6	5.5	6.3	2.4	510
	b) 1.7	5.2		1.9	328
	c) 2.4	4.4	2.7	1.6	244
Cat IV	a) 4.0	1.65	—	1.68	136
	b) 4.44	1.59	2.5	1.82	121
	c) 3.05	2.82	2.5	1.10	69.8
	d)		0.7	1.66	19.2
Cat V	4.0	7.5	7.8	0.89	34.1
Cat VI	3.5	12.5	12.1	1.0	59.6

The figures for the change in form of sulfapyridine in pancreatic juice and bile are given in Table 3.

The extremely high concentration of sulfapyridine in the bile of Cats III and IV coincides with the use of a slightly different drug solution.

Table 3.

Change of Sulfapyridine from Free to Bound Form in P. J. and Bile.

	Ratio of free to bound drug injected	Ratio of free to bound drug in Pan. Juice	Ratio of free to bound drug in Bile
Cat IV	13/87	—	0.41/99.6 (after 60 min.)
Cat V	5/95	6.5/93.7	4/96 (after 45 min.)
Cat VI	45/55	46/54	31/69 (after 45 minutes)

Results.

The blood level which was used as the standard of comparison varied in every cat, but exhibited a rapid decrease in concentration at first, followed by a very gradual decrease over a longer period. The concentration of sulfapyridine in pancreatic juice was slightly lower than in blood in every case except Cat VI, where this high concentration is probably due to an error in measuring the volume of juice. The result agrees with previous findings by other experimenters who were using secretions of pancreatic juice in dogs.

The concentration of the total drug in the bile was extremely high in every cat, much higher than would be expected from the work of CARRYER and IVY (1939) on dogs.

An increased flow of digestive juices can definitely be stated not to decrease the concentrations of sulfapyridine. At the same time, it is not possible to determine from our results if the converse of this is true. However, it would seem fairly clear that the concentration of drug in both pancreas and liver secretions is relatively independent of the rate of flow of the juices, and more dependent on the concentration level in the blood. Disregarding the concentration per unit volume of the drug in the pancreatic juice and bile, it is obvious that a very large total amount of the drug can be washed out on secretin stimulation of the two organs.

In connection with the change of sulfapyridine from the free to the bound form quantitative experiments were attempted in Cats IV and V with only qualitative results due to the insufficiency of free sulfapyridine in the injected preparation. The results nevertheless indicated that the relative percentage of free drug excreted in pancreatic juice remained unchanged while there was little or none excreted in the bile. In Cat VI, the 45 % free

drug solution was injected and 15 minutes after the last injection, the bile and pancreatic juice were collected over a half hour period. Analysis showed that in the pancreatic juice 46 % of the total drug was still free, or that there had been no change. In the bile however, only 31 % of the total drug was unbound, indicating the acetylation of 30 % of the free drug over a period of 45 minutes. It is very likely that acetylation of the drug takes a longer time due to the rapid washing when larger flows of bile are stimulated by secretin injection.

Summary.

The combined treatment of cats with sulfapyridine and secretin is described. In conformance with other investigations, in which secretin was not used, the concentration of sulfapyridine in pancreatic juice was found to be just lower than that of blood, but an extremely high concentration of the drug was found in the bile. The drug is not diluted by the increased flow of juice either from pancreas or liver and tends to be independent of rate of flow. Blood concentration of sulfapyridine is more important. While there was no change in the percentage of free drug excreted in the pancreatic juice, 30 % of the free drug had been bound when excreted in the bile, indicating acetylation by the liver.

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Some Quantitative Data on the Antagonism between Piperido-Methyl-Benzo-Dioxane (933F) and Adrenaline.¹

By

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According to a modern aspect on the mode of action of adrenaline, the site of action of this drug is attributed to certain receptors. The action of the drug involves two phases, the combining of the drug molecules with the receptors and the exertion of action after they have combined. This hypothesis, developed by CLARK, is supported by the fact that the concentration-action curve of adrenaline follows LANGMUIR-HITCHCOCK's law² (vide CLARK, 1936). Drugs which are specifically antagonistic to adrenaline are considered to have the ability of combining with the adrenaline receptors, thus blocking receptors to this drug, but unlike adrenaline the antagonistic drugs have no or little power of giving an effect after being combined with the receptors. In the case of certain antagonists it has already been made probable that the affinity between the receptors and adrenaline, respectively between the receptors and antagonist, is governed by the law of mass action, *i. e.* the presence of the antagonist does not alter the shape of the concentration-action curve, which still follows LANGMUIR-HITCHCOCK's law.

¹ Received 6 June, 1940.

² $k \cdot x = \frac{y}{100 - y}$; k is a constant, x is the concentration of the drug producing the effect y ; y is calculated as percentages of the maximal effect which can be produced by the drug in question.

The experiments of BACQ and FRÉDÉRICQ (1935) suggest that CLARK's hypothesis on drug antagonism may be applied to the antagonism between 933 F and adrenaline. Their results seem to indicate that 933 F inhibits the action of adrenaline by interfering with the specific receptors. The authors have, however, studied the antagonism only within a narrow range of concentrations of the drugs, which must be said to limit the possibilities of drawing conclusions.

In his article in *Physiological Reviews* (1937), ROSENBLUETH has another aspect on this antagonism. He tries to explain the fact that 933 F antagonizes adrenaline added to an organ, while it does not inhibit the effect of the stimulation of the sympathetic nerves of the same organ. He assumes that 933 F renders the cells impermeable to adrenaline, thus blocking its passage to its site of action, while the transmitter of sympathetic nerves is liberated within the cells in close relation to the site of action. According to this view, 933 F does not exert any action on the adrenaline receptors themselves. A confirmation of BACQ and FRÉDÉRICQ's opinion may thus have a certain interest, and we have, therefore, studied the quantitative relations of the antagonism of 933 F to adrenaline in experiments on perfused ears of rabbits.

In the case of the antagonistic drugs which are supposed to act on the specific receptors the quantitative data follow a simple expression, developed by GADDUM (1937) on the basis of LANGMUIR-HITCHCOCK's law and originally applied to the antagonism between acetylcholine and atropine:

$$K_1 [\text{Adr}] = (1 + K_2 [933 \text{ F}]^n) \frac{y}{100 - y};$$

K_1 and K_2 are constants; y is the effect which is produced by the concentration of adrenaline [Adr] in the presence of the concentration of [933 F]. The exponent, n , expresses the number of molecules of the drug which combine with one receptor. In our case the value of n was found to be 1, *i. e.* one molecule of the drug combines with one receptor. As we always determined the amounts of adrenaline giving

the same effect, the factor y was kept constant. The expression can then be simplified to:

$$K_1 [\text{Adr}] = (1 + K_2 [933 \text{ F}]) \cdot a;$$

The experiments were made on rabbits' ears, which were perfused through the artery with Ringer's solution. By means of Mariotte's flasks the perfusion pressure was kept constant at 350 mm. of water. The arrangements made it possible to shift from Ringer to 933 F solutions without interrupting the perfusion and without changes in the pressure. The rate of perfusion was measured with ROTHLIN's "Ordinatenschreiber", which automatically records the number of drops per minute. The various amounts of adrenaline, always dissolved in the same amount of Ringer solution, were injected in the connecting tube immediately before the cannula, inserted into the artery. In this arrangement those amounts of adrenaline were determined which produced a certain decrease of the drop rate before and after the addition of various amounts of 933 F. The results are seen from the table below.

The values experimentally found are in good accordance with values, calculated according to the expression above. The experiments, therefore, seem to indicate that the molecules of adrenaline and of 933 F react with the receptors in accordance with GADDUM's expression. Thus, the experiments support the observations of BACQ and FRÉDÉRICQ.

	Concentration of 933F (γ per ml of perfusing fluid)	0	0.01	0.1	1	10	100	1,000
	γ of adrenaline pro- ducing the same decrease of drop rate							
Experiment 1	a) observed . .	0.003	0.006	0.012	0.1	1.0	9	85
	b) calculated . .	—	0.004	0.012	0.09	0.9	9	90
Experiment 2	a) observed . .	1	.	3.0	25	300	2,500	.
	b) calculated . .	—	.	3.5	26	250	2,500	.

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Wirkung der Sauerstoffatmung auf die Atmungs- steigerung bei Carotisabklemmung.¹

Von

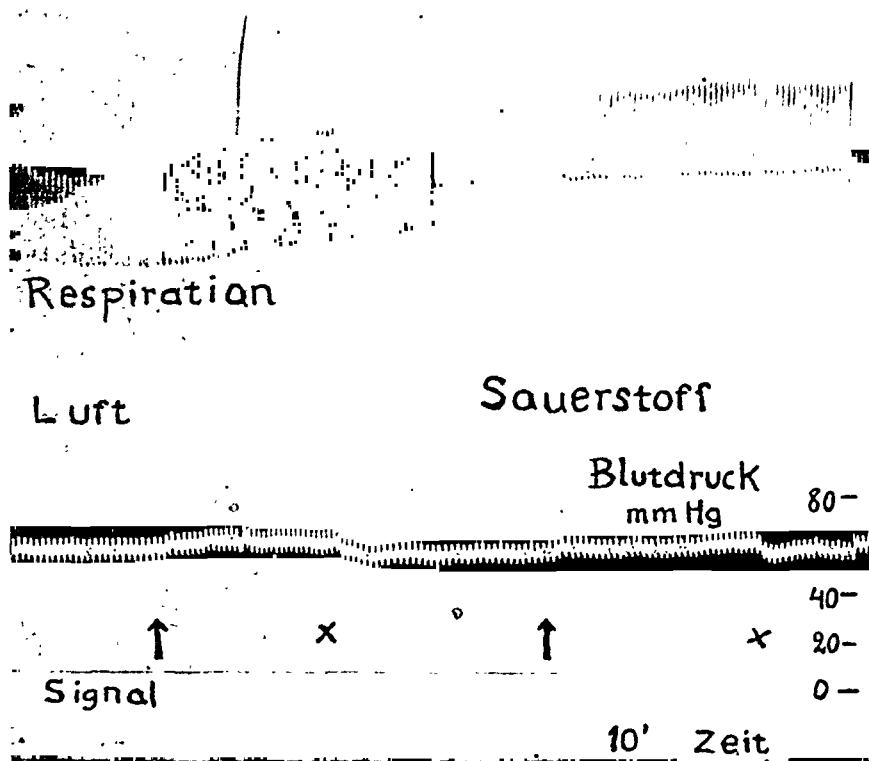
TURE RUDBERG.

(Mit 1 Abbildung im Text.)

In einer früheren Arbeit (RUDBERG 1938) wurde gezeigt, dass die ventilationssteigernde Wirkung der Carotidenabklemmung bei Katzen und Kaninchen wesentlich verstärkt wird, wenn der arterielle Blutdruck des Tieres unter einen gewissen kritischen Wert, der individuell verschieden ist, gefallen ist. Es wurde ferner die Vermutung ausgesprochen, dass dieser atmungserregende Effekt von einer Hypoxämieerregung des Sinus caroticus abhängen könnte. Es war deshalb von Interesse zu prüfen, ob Einatmung von Sauerstoff den erwähnten Effekt beeinflussen würde.

Solche Versuche sind jetzt an vier Katzen ausgeführt worden. Die Tiere wurden mit Urethan narkotisiert, worauf Blutdruck und Atmung registriert wurden. Die Atmung wurde in einem Falle mit Pneumograph, in den übrigen dagegen quantitativ mit Körperplethysmograph (vgl. v. EULER und LILJESTRAND 1936) aufgezeichnet. In zwei von diesen Fällen ist die Carotisabklemmung durch spezielle Klemmen, die die ganze Zeit rings um die Gefäße in fixierter Lage befestigt waren, durchgeführt worden. Hierdurch wurde die Ziehung bei der Abklemmung vermieden, und der Eingriff konnte geschehen, ohne dass der Plethysmograph geöffnet werden musste. Die Tiere atmeten durch Müllerventile. Durch Blutentnahme wurde der Blutdruck gesenkt, bis die Carotidenabklemmung kräftige Vermehrung der Atmung bewirkte. Jetzt wurde statt Luft Sauerstoff inspiriert; nach einer

¹ Der Redaktion am 22. August zugegangen.



Katze 1. Die Kurven bedeuten von oben Ventilation, Blutdruck, Signal und Zeit (10 Sek.). Durch Carotisabklemmung (zwischen ↑ und ×) wird die Ventilation bei Luftatmung um 48 Proz., bei Sauerstoffatmung um 11 Proz. vermehrt.

Vorperiode von einigen Minuten wurden die Carotiden nochmals abgeklemmt und zwar während etwa derselben Zeitspanne wie in dem früheren Versuch (gewöhnlich 1—2 Minuten). Hierbei ist der Blutdruck so gut wie unverändert geblieben. Der Versuch ist mehrmals an demselben Tier ausgeführt worden.

Es hat sich in sämtlichen Fällen gezeigt, dass die Atmungssteigerung nach Carotidenabklemmung wesentlich langsamer eintrat und kleinere Werte erreichte, wenn das Tier Sauerstoff geatmet hatte als bei Luftatmung. Der Unterschied ist in verschiedenen Fällen ungleich gross, aber immer ganz deutlich gewesen. Die Abb. 1 gibt einen typischen Versuch wieder, und in der Tabelle 1 werden die quantitativ ermittelten Werte zusammengestellt.

Es scheint also, als ob die Sauerstoffatmung durch bessere Sättigung des Hämoglobins mit Sauerstoff die atmungssteigernde

Tabelle 1.

Versuchstier	Inspirations- luft	Blut- druck mm Hg	Ventila- tions- Ver- mehrung in Proz.	Zeit, während welcher der Vergleich geschehen ist	Bemerkungen
Katze 1. .	Zimmerluft	60	48	} 40 Sek.	Carotidenabklem- mung mit spe- ziellen Klemmen
	Sauerstoff	58	11		
	Zimmerluft	60	37		
	Sauerstoff		14		
Katze 2. .	Zimmerluft	37	20	} 20 Sek.	, , ,
	Sauerstoff	35	10		
Katze 3. .	Zimmerluft	64	30	} 60 Sek.	
	Sauerstoff	62	7		

Wirkung der Carotidenabklemmung entgegenwirken kann. Dass das Blut bei den niedrigen Blutdruckwerten, die hier in Frage kommen, nicht mit Sauerstoff gesättigt ist, wurde auch direkt festgestellt. Es wurden zu diesem Zwecke arterielle Blutproben teils bei einem Blutdruck, wo die Carotidenabklemmung nur wenig die Ventilation steigerte, teils auch unterhalb des kritischen Druckes entnommen und nach van Slykes manometrischer Methode analysiert. Die Tabelle 2 gibt die Ergebnisse.

Tabelle 2.

Versuchstier	Blut- druck mm Hg	Sauer- stoff- gehalt Vol. %	Sauer- stoff sättigung in %	Wirkung der Carotiden- abklemmung auf die Atmung	Bemerkung
Katze 4. .	76	16.8	100	schwach	kritischer Druck wahrscheinlich zwischen 68 und 56 mm
	68	16.8	100	schwach	
	56				
	54	16.0		gut	
	52				
Katze 5. .	50	15.5	93.4	gut	
	60	18.1	96.4	schwach	
	41	16.0	82.8	gut	

Aus den Versuchen muss geschlossen werden, dass wenigstens ein wesentlicher Teil der Ventilationsvermehrung, die durch Carotidenabklemmung bei der Katze mit relativ niedrigem Blutdruck erhalten wird, durch eine Hypoxämieerzierung zustande kommt. Das Ergebnis ist in guter Übereinstimmung mit dem Befund,

dass bei der Carotisabklemmung eine vermehrte Anzahl von Aktionspotentialen aus den Nervenfasern der Chemoreceptoren des Sinus caroticus nachgewiesen werden kann (v. EULER, LILJESTRAND und ZOTTERMAN 1939).

Zusammenfassung.

An der Katze wird gezeigt, dass der ventilationsvermehrenden Wirkung der Carotidenabklemmung bei relativ niedrigem Blutdruck durch Einatmung von Sauerstoff entgegengewirkt werden kann. Ein wesentlicher Teil wenigstens der Atmungssteigerung muss deshalb durch Hypoxämieerregung des Sinus caroticus zustande kommen.

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The Effect of Carotid Sinus Denervation on Respiration during Rest.¹

By

U. S. v. EULER and G. LILJESTRAND.

The discovery by HEYMANS, BOUCKAERT and DAUTREBANDE (1930) that the chemoreceptors of the carotid body are stimulated by hypoxemia, as well as by hypercapnia, thereby increasing respiration reflexly, raised the problem whether such reflexes play any rôle under physiological conditions or may be considered only as part of an emergency mechanism. It was soon generally agreed that oxygen lack stimulates respiration practically only over the sinus mechanism, whereas opinions still differ with regard to carbon dioxide. Thus v. EULER and LILJESTRAND (1936) and HEYMANS and BOUCKAERT (1939) came to the conclusion that carbon dioxide influences respiration normally by the reflex mechanism mentioned; SCHMIDT and COMROE (1938), on the other hand, consider that the sinus mechanism hardly takes any part in this regulation. Investigations on the action potentials from the sinus nerve have shown conclusively, as far as we can judge, that even a small increase in the desaturation of the hemoglobin or of the carbon dioxide tension of the blood will cause a considerable rise in the number of potentials (SAMAAN and STELLA 1935, v. EULER, LILJESTRAND and ZOTTERMAN 1939). It was also observed that such potentials are elicited already at a hemoglobin saturation with oxygen of about 95 per cent or a carbon dioxide tension of about 30 mm respectively. These observations would seem to indicate that the reflexes concerned probably act under ordinary conditions, but

¹ Received 3 September 1940.

they tell us nothing with regard to their quantitative significance.

The simplest way of determining this is undoubtedly to compare respiration before and after denervation of the sinuses. If respiration during rest is normally to some extent stimulated from the sinus region, then it is to be expected that denervation will reduce the ventilation. The possible compensation from the aorta nerves, analogous to that which occurs with regard to the blood pressure effect of sinus denervation, seems to be of little significance for the respiration. There is, however, another complication, since the denervation has an indirect stimulating effect on respiration, which is quite independent of any normal direct chemoreflex over the respiratory centre. It was found by v. EULER and LILJESTRAND (1935) that clamping the carotids in the dog resulted in an increased metabolism. The rise in the oxygen consumption varied considerably; on an average it was 11 per cent. MERTENS and REIN (1938) confirmed this result, though the increase observed by them was smaller. HAHN (1940), on the other hand, after section of both the sinus and aorta nerves in the dog, found a rise of 10—35 per cent in the oxygen consumption. As is well known, increased metabolism causes a rise in ventilation, and there is no reason why this should not be true in cases where the rise in metabolism is brought about by sinus denervation. It is to be expected, however, that the alveolar carbon dioxide tension will not rise appreciably with a moderate increase in metabolism. If, now, denervation of the sinuses does away with reflexes which normally stimulate respiration, then the increase in ventilation from the rise in metabolism may become more or less compensated, or the result may even be increased ventilation. A definite index of the significance of the reflexes concerned will, however, be a rise in the alveolar carbon dioxide, indicating that the centre alone is unable to maintain it at the usual level. This increase above the value before denervation will therefore give a clear expression of the rôle of the chemoreflexes under physiological conditions.

A decrease in ventilation after denervation of the sinuses has been observed by several investigators. Thus, in 4 cats under chloralose anesthesia, SELLADURAI and WRIGHT (1932) found an average decrease of 22 per cent. In 6 decerebrated cats the corresponding average lowering was 33 per cent. It must be observed, however, that the carotids were open only in one of these

cases (with a decrease in ventilation of only 5 per cent). Since the ligaturing of the carotids gives rise to oxygen want, with a corresponding increase in ventilation, which is abolished by the denervation, these last-mentioned experiments give no satisfactory evidence as regards the normal participation of the sinus mechanism in the regulation of respiration. In dogs, cats and rabbits WITT, KATZ and KOHN (1934) observed a respiratory standstill, and death after careful denervation of the sinus region. They conclude that a tonic influence on respiration is exercised from the sinus region. v. EULER and LILJESTRAND (1936) found in anesthetized cats (chloralose) a rise in the alveolar carbon dioxide of some 1.5 per cent, indicating a decrease in ventilation of about 25 per cent. In anesthetized dogs GESELL and LAPIDES (1938), as well as SCHMIDT, COMROE and DRIPPS (1939), observed depressed respiration in consequence of the denervation. SCHMIDT, DUMKE and DRIPPS (1939), experimenting on lightly anesthetized dogs, whose carotid pressoreceptors had been divided, while the chemoreceptors were left functioning, found that blocking of the sinus nerves with prokain during oxygen inhalation did not consistently affect respiration. It ought to be remarked that the dogs had received morphia, which is known greatly to affect the respiratory centre.

The evidence quoted above has recently been reviewed by SCHMIDT and COMROE (1940). They maintain that anesthesia may greatly influence the respiratory centre, whereas the chemoreceptors remain effective; they further point out that the denervation may be accompanied by trauma, affecting respiration, and that the rise in blood pressure following the denervation may be responsible for changes in breathing. There is also the possibility that a decreased ventilation after sinus denervation may, wholly or in part, be the consequence of some oxygen want which will stimulate respiration over the sinus mechanism, but not directly on the centre. According to SCHMIDT and COMROE the above-mentioned experiments by SCHMIDT, DUMKE and DRIPPS are free from these objections.

It is quite clear that the use of an anesthetic will depress the respiratory centre more or less. We think, however, that the effect can be greatly reduced if a suitable anesthetic is chosen and the dose given is not too large. It is probably possible to find a point where the cortex is eliminated, though the centres of the medulla are hardly affected. The animal will not then

display the great lability of the untrained unanesthetized or of the lightly anesthetized animal, and small sensitive stimuli will have no influence on respiration. If the depression of the respiratory centre is noticeable, one must expect that the alveolar carbon dioxide tension will rise. We have shown before (1936) for cats — and similar results were obtained for dogs, though they were not reported in detail — that the alveolar carbon dioxide was held for hours about 5—6 per cent of an atmosphere, when the animal had been anesthetized with chloralose (6—10 cg per kg of body weight). This completely tallies with the values found by ROOS and ROMIJN (1937) for the trained unanesthetized dog. As for cats no direct observations without anesthesia are known, but there is good reason to believe that the alveolar carbon dioxide tension is similar to that of the normal dog. If sleep is beginning to become superficial, a drop in the carbon dioxide tension marks that the experimental conditions act as a sensitive stimulus. A light anesthesia, as used by SCHMIDT, DUMKE and DRIPPS, may therefore easily involve special difficulties in obtaining constant results.

There can be no doubt that a trauma may greatly influence respiration. The type of trauma involved in our experiments will always — with the exception of transitory effects of very short duration — produce a stimulation of respiration. Consequently a depressing effect of the sinus denervation may to some extent become obscured by the trauma. That the decrease in respiration after the denervation which has been observed by several investigators is to be explained by the effect of the unspecific trauma is in our opinion very unlikely, and no proof of this assumption has been given.

With regard to the possible effect on respiration of the rise in blood pressure following the denervation, the evidence available does not indicate any such effect. HEYMANS and BOUCKAERT (1930), after having cut the depressor and sinus nerves in dogs, observed no change in respiration after the intravenous injection of 0.3 mg adrenaline, which produced a considerable rise in the blood pressure.

The rise in alveolar carbon dioxide after sinus denervation was at first interpreted as a proof that carbon dioxide stimulated respiration, in part reflexly, under normal conditions (EULER and LILJESTRAND 1936). The possibility that the effect might to some extent be due to the absence of a stimulation from oxy-

gen lack was excluded by the observation that a corresponding rise in alveolar carbon dioxide was observed, even if the animal was breathing oxygen. The results quoted above concerning the action potentials in the sinus nerve have demonstrated, however, that even a very small degree of oxygen lack may stimulate the nerve endings. It is therefore quite likely that a simultaneous effect of oxygen lack and carbon dioxide on the sinus mechanism occurred in our earlier experiments, though carbon dioxide alone was responsible for the result in the controls mentioned. In order to determine the effect of carbon dioxide alone it became desirable to make experiments while the animal was breathing oxygen.

Methods.

We have used cats and dogs in our experiments. They were usually anesthetized with chloralose (0.06—0.10 g per kg of body weight). In the dogs a small amount of barbiturates (0.1 ml somnifen per kg) was added, in order to reduce shivering. Some experiments were performed with cats decerebrated under ether anesthesia. Since it was necessary to maintain the carotids intact, they had to be kept open, and this involved a great risk of hemorrhage. Among several methods tried, we found the intravenous injection of sangostop (2 ml of a 5 per cent solution per kg of body weight) before the decerebration the most effective means of preventing bleeding. The animal breathed through Müller or Lovén valves. Alveolar air samples were obtained through a fine catheter, inserted in the side-tube of a tracheal cannula. The lower end of the catheter reached the bifurcation of the trachea, and an airtight connection was established with the cannula. A few ml of alveolar air were drawn into a sampling tube at the end of each respiration. Ventilation was recorded quantitatively with the aid of the body plethysmograph described in an earlier paper (EULER and LILJESTRAND 1936). Denervation of the sinuses was made by mass ligation of the carotid body, the bulk of tissue between the external and the internal carotids being ligatured, after the region concerned had been carefully dissected free before the experiment started. The reaction of the chemoreceptors before and after denervation was tested by letting the animal inspire a gas mixture of 7 per cent of oxygen in nitrogen.

Results.

The following abbreviated record will serve as a type experiment.

6. 3. 1940. Cat, 4.8 kg, receives under ether anesthesia 35 ml of a 1 per cent solution of chloralose intravenously. Tracheal cannula. Sinus region dissected. Animal in body plethysmograph. Blood pressure recorded from femoral artery.

Time	Ventilation l. per min.	Respiration rate per min.	Alveolar carbon dioxide per cent	Blood pressure mm Hg
10.47	0.68	13.9	6.40	141
10.56	0.69	14.0		152
10.57	Inspires oxygen.			
11.03	0.65	13.2	6.69	154
11.11	0.70	14.0	6.57	166
11.13	Inspires air.			
	0.76	14.7		176
	0.76	15.0	6.46	180
11.27	Inspires oxygen.			
11.31	0.68	14.2		173
11.34	0.73	14.6	6.46	172
11.36	Inspires air.			
11.37	0.74	14.7		180
11.39	0.78	15.4		181
11.45	7 per cent of oxygen in nitrogen gives the typical increase in ventilation. Denervation of both sinuses. Inspires air.			
12.18	0.55	12.3	7.53	223
12.22	7 per cent of oxygen in nitrogen gives practically no increase in ventilation. Inspires air.			
12.38	0.57	12.6		170
12.45			7.82	
13.10	0.59	12.8	7.77	143
13.14	Inspires oxygen.			
13.17	0.56	11.7	7.78	182
13.20	Inspires air.			
13.21	0.54	11.2		185
13.25	0.57	12.9		131

The anesthesia in this case was probably rather deep, and consequently the animal had a relatively low ventilation and high alveolar carbon dioxide pressure. There seems to be a tendency for ventilation to increase as time goes on, probably as a result of a gradual fall of the depth of anesthesia. When oxygen is given before denervation, a small decrease in ventilation is

observed during the next few minutes, indicating that some oxygen lack exists. A compensation takes place later on. Denervation gives the typical effect: ventilation decreases by about 27 per cent, and alveolar carbon dioxide rises by about 20 per cent. Oxygen is now without effect. In this case — as in our earlier control experiments — the effect of denervation is quite obvious, even if the animal has been breathing oxygen, so that complete saturation of the hemoglobin is ensured. The conclusion is that the decrease in ventilation after denervation is due to the loss of the chemical stimulation of the carotid body by the normal carbon dioxide tension of the blood. The blood pressure rose immediately after the denervation but soon fell again to the old level. A changed blood pressure cannot therefore be responsible for the changed respiration.

Similar results have been obtained with an alveolar carbon dioxide pressure somewhat below the physiological level, as illustrated by the following experiment.

21. 2. 1940. Cat, 4.5 kg, receives under ether anesthesia 35 ml of a 1 per cent solution of chloralose and 4 ml of a 20 per cent solution of urethane intravenously. Preparation as above.

Time	Ventilation l. per min.	Respira- tion rate per min.	Alveolar carbon dioxide per cent	Blood pressure mm Hg	Arterial blood gases	
					O ₂ per cent	CO ₂ per cent
0'	1.20	20.2	4.88	190	17.1 (89.5 % sat.)	36.2
5'	Inspires oxygen.					
6'	1.10	19.7	4.88	192	20.5 (100 % sat.)	33.6
12'	Inspires air.					
15'			4.61			
18'	7 per cent oxygen in nitrogen gives considerable rise in ventilation.					
30'	Both sinuses denervated. 7 per cent oxygen in nitrogen gives a small increase in ventilation.					
35'	Inspires air.					
37'	Inspires oxygen.					
39'	0.87	17.3	5.00	183		
42'	Inspires air.					
45'	0.80	17.4	5.17	184		

The decrease in ventilation after denervation is of the usual order, though the increase in alveolar carbon dioxide is relatively small. It is possible that this was due to the fact that denervation was not absolutely complete. As in the case of the former

Table
Decerebra-

Date	Weight kg	Before denervation							
		Breathing air				Breathing oxygen			
		Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg	Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg
³ / ₄ 40	2.4	1.35	34.5	—	—	0.93	28.8	4.01	—
		—	—	—	—	1.06	30.0	—	—
⁴ / ₅ 40	2.5	1.85	37.2	3.63	62	1.70	36.2	3.72	74
		¹ 1.18	13.0	4.12	59	—	—	—	—
³ / ₅ 40	2.7	1.17	30.8	3.83	112	1.18	30.0	4.02	114
		—	—	—	—	1.14	30.0	3.74	112

experiment, this also demonstrates that the effect is obtained, even if the animal is breathing oxygen. A rise in the blood pressure can obviously not be regarded as a determining factor for the decrease in ventilation in this case.

Decerebration has the advantage that anesthesia is rendered unnecessary, but it certainly involves a very heavy traumatization of the central nervous system, which will often greatly affect respiration. Sometimes after decerebration we have observed different signs of respiratory disturbances, such as periodical breathing, which is more or less alleviated by oxygen inhalation, gasps etc. Sometimes we have seen a continuous increase in respiration, which must be ascribed to some stimulus of unknown origin. Thus the alveolar carbon dioxide may show very low values. We have observed, e. g., 1.38 and even 0.8 per cent. Obviously such animals are unsuitable for experiments of the kind in question, which aim at testing the effect of denervation of the sinuses. We have succeeded, however, in reaching results with decerebrated cats where respiration goes on with sufficient constancy for our purpose. Table 1 gives the results of three such experiments.

The results agree with those obtained under anesthesia. Oxygen inhalation before the denervation reduces ventilation in every case, but there is a further decrease after denervation. Alveolar carbon dioxide percentage then rises by 0.4—0.9.

¹ After vagotomy.

1.

ted cats.

After denervation							
Breathing air				Breathing oxygen			
Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg	Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg
—	—	—	—	—	—	—	—
0.90	31.2	5.09	—	0.90	30.0	4.92	—
—	—	—	—	—	—	—	—
—	—	—	—	0.85	7.4	4.98	48
—	—	—	—	—	—	—	—
—	—	—	—	1.05	32.5	4.18	86

SCHMIDT, DUMKE and DRIPPS (1939) have pointed out that dogs and cats might react differently to denervation of the sinuses. Even though this is not very probable, it seemed desirable for us to perform some experiments with dogs under the same experimental conditions as those prevailing during the observations on cats. Our results are summarized in table 2.

In experiments 1, 2 and 3 no oxygen was given, and we cannot, therefore, exclude an influence of relative oxygen lack. There is a decrease in ventilation after denervation of 26, 11 and 14 per cent respectively. In the following experiments oxygen was given at intervals, allowing us to compare the reactions with, as well as without, oxygen. In experiment 5 the denervation was incomplete, as was shown by the reaction to 7 per cent oxygen in nitrogen. This explains why oxygen is able to reduce the ventilation, not only before, but also after the denervation, which otherwise does not occur. Both values, however, show a small decrease (5 and 7 per cent) in relation to the corresponding values before denervation, and a corresponding rise (6 per cent) is observed in the alveolar carbon dioxide percentage. In experiment 4 oxygen produced a very small reduction of ventilation before denervation. When this had been performed, the ventilation was reduced by 14 and 13 per cent respectively, and the alveolar carbon dioxide rose by 28 per cent. Experiment 6 showed a great decrease (24 %) in ventilation, when oxygen was given, but there was a further reduction (12 % of the

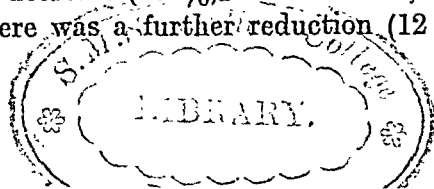


Table
Anestheti-

Exp. nr. Date	Weight kg	Before denervation							
		Breathing air				Breathing oxygen			
		Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg	Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg
1938									
1 ¹⁶ / ₁₁	13.5	7.27	19.4	—	77	—	—	—	—
2 ³ / ₁₂	28	7.43	22.8	—	78	—	—	—	—
3 ¹⁴ / ₁₂	12.5	3.76	23.0	4.56	104	—	—	—	—
1940									
4 ¹⁵ / ₅	13.5	5.14	22.0	4.03	—	5.06	22.0	4.01	140
5 ²⁰ / ₅	13.5	—	—	—	—	2.72	14.0	6.44	112
		3.57	15.9	—	114	—	—	—	—
6 ¹ / ₆	29	10.29	22.8	4.61	134	—	—	—	—
		—	—	—	—	7.86	18.7	4.78	120
7 ⁵ / ₇	18	7.57	34.0	5.34	132	—	—	—	—
		—	—	—	—	5.05	26.9	5.53	134
		8.77	37.6	—	137	—	—	—	—
		8.12	36.5	4.76	140	—	—	—	—
		—	—	—	—	7.95	39.6	4.60	134

original value) when denervation was performed. The corresponding rise in the alveolar carbon dioxide was, in both cases, much smaller than one would expect from the effect on ventilation. In experiment 7 oxygen given at the beginning of the experiment caused a very pronounced reduction in ventilation; later on ventilation seemed to increase spontaneously, and simultaneously the usual effect of oxygen — a reduction of ventilation — was to a great extent reduced. Still the denervation had the usual effect, the reduction in ventilation being 26 and the increase in alveolar carbon dioxide 24 per cent.

The rise in the blood pressure was moderate in all the experiments where vagotomy was not performed, and it does not seem very probable that this rise can explain the effect on respiration.

Our observations on dogs are in complete harmony with the results obtained with cats. For both types of animals we have

2.

zed dogs.

After denervation							
Breathing air				Breathing oxygen			
Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg	Ventila- tion l. pr. min.	Resp. rate pr. min.	Alveol. CO ₂ perc.	Blood press. mm Hg
5.35	11.5	—	68	—	—	—	—
6.64	27.0	—	96	—	—	—	—
3.23	20.0	5.26	103	—	—	—	—
4.40	22.0	—	—	4.40	22.0	5.14	176
—	—	—	—	—	—	—	—
—	—	—	—	² 2.53	13.6	6.85	144
¹ 3.39	15.9	6.75	150	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	6.58	15.9	5.06	152
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	5.86	30.0	5.70	172

been able to demonstrate, in confirmation of earlier experiments on cats, that denervation of the sinuses leads to a decreased ventilation and an increased carbon dioxide percentage of the alveolar air.

Summary.

After denervation of the sinuses, a decrease in ventilation and an increase in the alveolar carbon dioxide percentage was observed in cats and dogs. The effect was reduced but not abolished, if the animals were breathing oxygen. Neither anesthesia nor increase of the general blood pressure can explain the effect. It is concluded that the carbon dioxide tension of the blood under

¹ Both vagi cut.

² Denervation incomplete.

physiological conditions stimulates respiration, not only by a direct action on the respiratory centre, but also reflexly over the sinus mechanism.

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The Interaction of Amino Nitrogen and Carbohydrates.¹

By

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During the investigation of certain reactions in cell-free liver-extracts (ÅGREN 1940) an interaction of carbohydrates with free amino groups was observed. When reaction had ceased the decrease in the VAN SLYKE-determinable nitrogen was not accompanied by a decrease in titrable amino nitrogen; neither was any loss in total nitrogen or of ammonia noted even with an 80 % disappearance of VAN SLYKE nitrogen.

This great reactivity by incubation at 37° and pH 7.4 without any loss in ammonia was rather surprising when compared with earlier observations in model experiments with amino acids and sugars. MAILLARD (1912) and later BORSOOK and WASTENEYS (1925), EULER and others (1926) showed that the interaction between glucose and amino acids as alanine and glycine proceeded at a slow rate and to a limited extent at temperatures between 20° and 40° and pH 7. In alkaline solution the rate of reaction rapidly increased with the rise of temperature and pH. During this process ammonia was produced. BORSOOK and WASTENEYS in accordance with FEARON and MONTGOMERY (1924) in explaining the effect of the aldehyde group suggested a disturbance of the equilibrium of the amino group by its preliminary combination with the aldehyde group. NEUBERG and others (1927) noticed a speedy reaction at pH 7 between keto-sugars (fructose, hexose-diphosphate) and amino acids as ala-

¹ Received 7 June 1940.

² Fellow of the Rockefeller Foundation 1938—1939.

nine, aspartic acid and glutamic acid. The rapid interaction between keto-sugars and amino groups did not produce any further effect than that of the same amino acids and aldehydes.

For further investigation regarding the reaction in the liver extracts the model experiments were extended to the usual amino acids and carbohydrates incubated with phosphate to a concentration observed in the liver extracts (ÅGREN 1940).

Experiment.

Solutions of amino acids and sugars were made in glass-distilled water containing glucose in a concentration of 0.5 M. Phosphate was added in a mixture of SÖRENSEN's phosphate buffers pH 7.4 to the neutralized amino acid-sugar solutions. The phosphate concentrations were M/15 and M/30 in two series of experiments. The concentration of easily soluble amino acids of 0.3 M or of practically saturated solutions was used. The pH level was maintained at 7.4 (glass-electrode) during all experiments. The amino nitrogen was followed with the VAN SLYKE method in 24 hour periods. The solutions were kept at N₂-atmosphere in THUNBERG tubes and incubated at 40° or sealed in tubes at 100°. Results of typical experiments are given in table 1.

A comparison of the free amino nitrogen values shows, in agreement with earlier observations, that the interaction at pH 7 between amino acids and glucose only takes place to a very limited extent. The reaction is accelerated by raising the temperature to 100° and increasing the phosphate concentration from m/30 to m/15. There seems to be an optimal concentration corresponding to about m/15. Raising the phosphate concentration to about m/2 did not increase the rate or the extent of the reaction. There is one especially reactive amino acid: cysteine. About 80 per cent of the free amino nitrogen in cysteine disappeared in 48 hours. In the experiment at 100° the well known instability of cysteine at this temperature interfered with the expected result.

The catalyzing effect of phosphate in the experiments was not very marked but evident and had already been proved in the liver extracts (ÅGREN 1940). In other experiments the influence of varying phosphate concentrations in nitrogen and oxygen

Table 1.

Loss of van Slyke nitrogen in the interaction between amino acids and glucose.

Amino acid	Phosphate concentration in mol.	Temperature	Loss of van Slyke nitrogen in per cent of zero value after			Total loss in 72 hours per cent
			24 hours	48 hours	72 hours	
Alanine 0.3 M. . .	M/30	40 C	5	4	0	9
	M/15	40 C	8	6	0	14
	M/15	100 C	10/30 minutes			
Glycine 0.3 M. . .	M/30	40 C	5	3	4	12
	M/15	40 C	7	3	4	14
	M/15	100 C	8/30 minutes			
Serine 0.3 M. . .	M/30	40 C	11	0	0	11
	M/15	40 C	11	4	0	14
	M/15	100 C	15/30 minutes			
Leucine 0.2 M. . .	M/30	40 C	8	5	0	13
	M/15	40 C	9	5	0	14
	M/15	100 C	12/30 minutes			
Valine 0.3 M. . .	M/30	40 C	7	8	0	15
	M/15	40 C	7	6	0	13
	M/15	100 C	15/30 minutes			
Cysteine 0.3 M. . .	M/30	40 C	23	23	21	67
	M/15	40 C	72	6	0	80
	M/15	100 C	35/30 minutes			
Glutamic acid 0.2 M.	M/30	40 C	8	0	0	8
	M/15	40 C	8	4	0	12
	M/15	100 C	21/30 minutes			
Arginine 0.3 M. . .	M/30	40 C	5	0	0	5
	M/15	40 C	8	4	0	12
	M/15	100 C	15/30 minutes			
Histidine 0.3 M. . .	M/30	40 C	8	5	0	13
	M/15	40 C	8	6	0	14
	M/15	100 C	21/30 minutes			
Tryptophan 0.08 M.	M/30	40 C	0	0	0	0
	M/15	40 C	0	0	0	0
	M/15	100 C	0/30 minutes			
Lysine 0.3 M. . .	M/30	40 C	7	8	0	15
	M/15	40 C	8	7	0	15
	M/15	100 C	21/30 minutes			

atmosphere was further investigated. The experimental conditions were the same as those described above. A typical series is given in table 2.

The catalyzing effect of phosphate is demonstrable both in aerobic and anaerobic conditions. In the phosphate-free solu-

Table 2.

Disappearance of van Slyke nitrogen in aerobic and anaerobic alanine-glucose solutions incubated with phosphate at 40° C and pH 7.4

Sample	Van Slyke nitrogen in mg/cc after			
	0 hours	24 hours	48 hours	72 hours
O ₂ + PO ₄	4.20	3.98	3.80	3.81
O ₂	4.20	4.15	4.10	4.12
N ₂ + PO ₄	4.20	4.05	3.90	3.88
N ₂	4.21	4.18	4.12	4.11

Concentration of alanine, glucose and phosphate respectively 0.3 M., 0.5 M. and 0.07 M. O₂ = oxygen, N₂ = nitrogen, PO₄ = phosphate.

tions the reaction was almost unnoticeable. As a comparison may be mentioned that a phosphate-free solution of arginine and glucose (0.3 M solution) kept at 40° and pH 10 showed a 28 per cent loss of VAN SLYKE nitrogen in 60 hours.

In alanyl-glycine the pK_b is about 10 times as high as in alanine. This difference in dissociation constant could eventually influence the reaction with the aldehyde groups. A 0.3 M solution of a racemic peptide preparation was incubated with glucose and phosphate at pH 7.4 to a final concentration of 0.5 M respectively M/25. The experiments were performed in THUNBERG tubes with oxygen atmosphere. The results of a typical series are given in table 3.

Table 3.

Decrease of van Slyke nitrogen in aerobic alanyl-glycine sugar solutions incubated with phosphate at pH 7.4 and 40° C.

S a m p l e	Van Slyke nitrogen in mg/cc after		
	0 hours	24 hours	48 hours
Phosphate	4.16	2.85	2.50
Control without phosphate	4.18	4.11	4.05

Concentration of alanyl-glycine, glucose and phosphate respectively 0.3 M. 0.5 M. and 0.04 M.

An increase in the dissociation of the amino group did not accelerate the reaction with aldehyde groups. The stimulating effect of phosphate ions was more obvious in these experiments than in the alanine-sugar experiments. Whether the phosphate is influencing the amino group-sugar association or indirectly stimulating the process, by way of the carboxylic groups, will be further investigated.

In some experiments the tendency to an association between amino groups and keto-sugars was examined. In the first series alanine was incubated with fructose and phosphate at 40° and pH 7.4, the final concentrations being respectively 0.3 M, 0.3 M and 0.1 M. There was no provable disappearance of VAN SLYKE nitrogen in 24 hours. Negative results were also obtained in other series with cysteine, cystine and alanyl-glycine. In this connection it should be emphasized that there was no difference in the reaction between natural and unnatural amino acids with aldehyde and keto sugars. The negative results with cysteine were surprising in view of the rapid and complete reaction of that amino acid with glucose. Next methylglyoxal as representative of ketoaldehydes was used. This was found to react instantaneously. The results obtained in the first VAN SLYKE determination immediately after solving the amino acids in the solution of methylglyoxal were not decreased in the next 48 hours, as demonstrated in table 4.

Table 4.

Disappearance of van Slyke nitrogen in aerobic methylglyoxal-amino acid solutions incubated at 40° C with phosphate. pH = 7.4.

Amino acid	Van Slyke nitrogen in mg/cc after		
	0 hours		24 hours
	Calc.	Found	
Alanine	2.80	2.50	2.40
Alanyl-glycine	2.80	2.40	2.39
Cysteine	2.80	0.55	0.50

Concentration of amino acids, methylglyoxal and phosphate respectively 0.2 M., 0.2 M., 0.04 M.

It was obvious from all experiments that the equilibrium in the reaction between amino and aldehyde groups at pH 7 was

with one exception very unfavourable from a preparatory point of view. Conclusively interest was focussed on the reaction between cysteine and aldehydes and eventually on the ketoaldehydes. In a solution of cysteine and glucose (0.3 M respectively 0.5 M) the reaction ceased when about 80 per cent of the VAN SLYKE nitrogen had disappeared. The results obtained then remained constant for weeks. When the molecular ratio cysteine

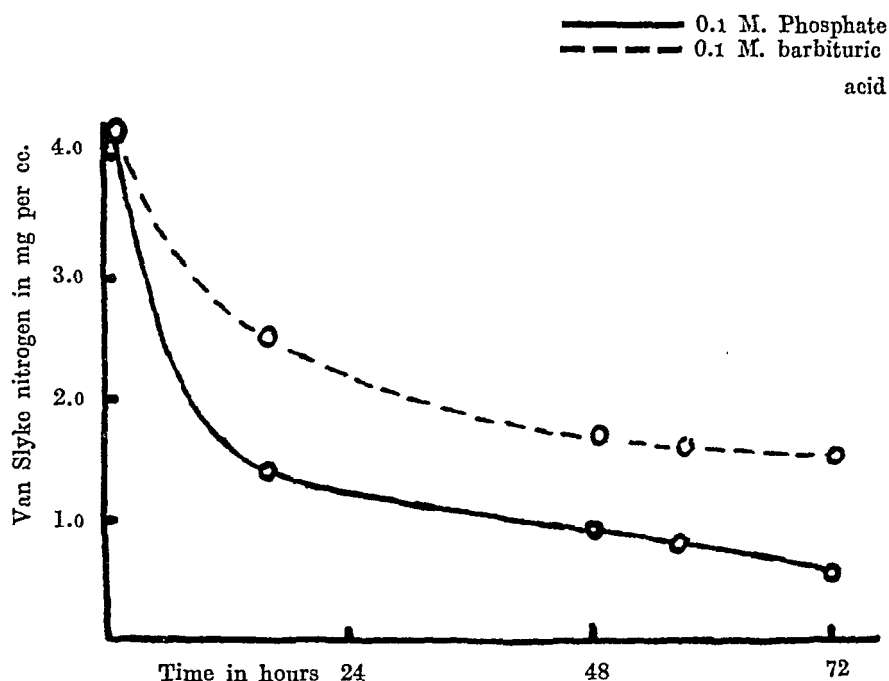


Figure 1.

Activating effect of phosphate on the cystein sugar reactions; pH 7.4, 40° C.

Concentration of cysteine and glucose respectively 0.3 M. and 0.5 M.

to sugar was 3 : 1 the VAN SLYKE nitrogen disappeared to a point corresponding to a complete reaction between sugar and cysteine. A surplus of cysteine could clearly force the reaction to completion but was, it seemed, an uneconomical way of preparation and afterwards equimolecular amounts of sugar and cysteine were used. It is a well known fact that the usual cysteine preparations contain small amounts of copper, which in the presence of air will oxidize cysteine to cystine. This was undesirable since experiments already had shown that cystine reacted in the same limited way as the other amino acids. In all cysteine-

sugar experiments glass-distilled water was used and eventually interfering copper was removed with diethyl-dithio-carbamide (AGNER 1939). THUNBERG tubes and nitrogen atmosphere excluded any oxygen influence. Determinations of cysteine and cystine were made according to the method of KASSEL and BRAND (1938) readings being made from standard curves of cystine and

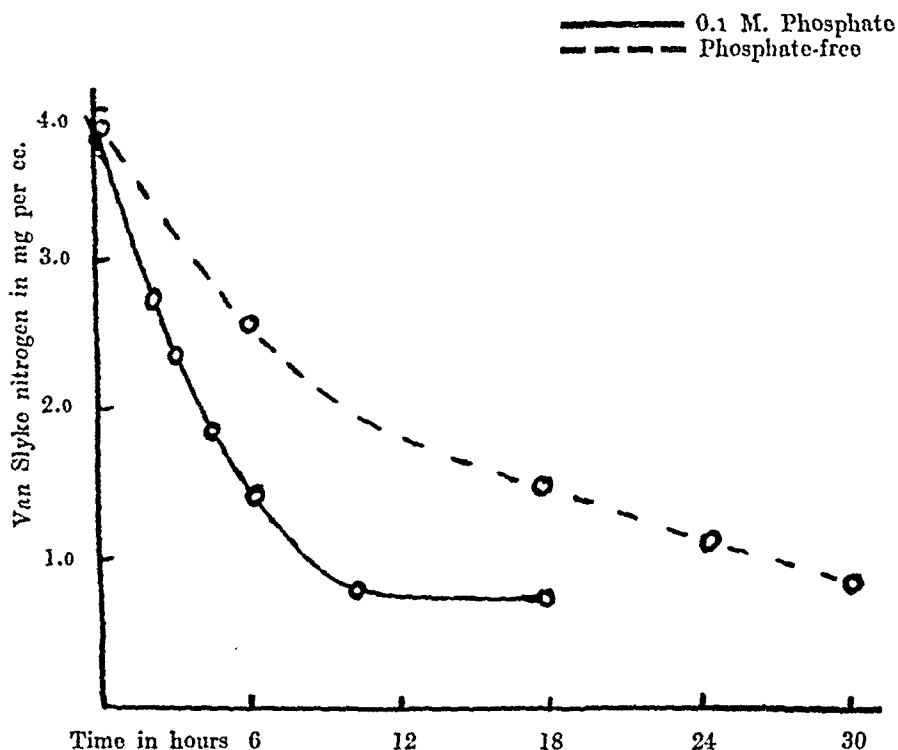


Figure 2.

Disappearance of VAN SLYKE nitrogen in cysteine-sugar solutions; pH constantly kept at pH 7.4 by adding 10 N NaOH. 40° C.

Concentration of cysteine and glucose respectively 0.3 M. and 0.5 M.

cysteine preparations used in the experiments. Glucose was determined with the orcinol method according to the modification of SÖRENSEN and HAUGAARD (1933), and phosphate according to LUNDSTEEN and VERMEHREN (1936). The optimal conditions of the reaction were studied in series of experiments. When the cysteine-glucose reaction was followed by VAN SLYKE determinations in 0.1 M solutions of phosphate and barbituric acid buffers (pH 7.5) the reaction became more definite in phosphate

solutions. (Figure 1.) The pH at the end of the experiment was in both solutions about 5.8.

In two series of experiments solutions of cysteine and sugar were constantly kept at pH 7.4 by adding 10 N NaOH with due precaution being taken to exclude air. Phosphate was present in one of the series in a final concentration of 0.1 M. The decrease in VAN SLYKE nitrogen is pictured in figure 2. The activating effect of phosphate ions was manifest.

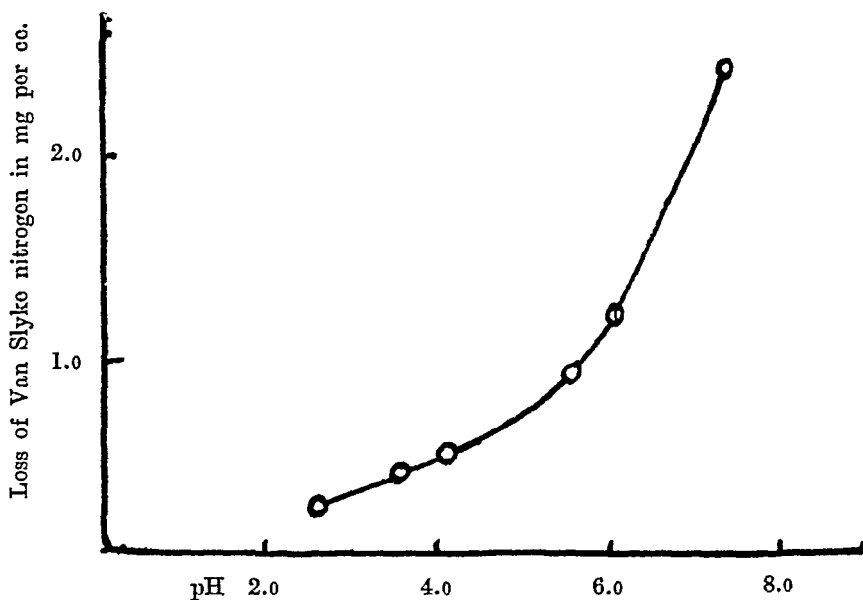


Figure 3.

Disappearance of VAN SLYKE nitrogen in anaerobic cysteine-glucose solutions incubated at 40° C. in 0.2 M. buffer solutions. Reaction time 24 hours. The values calculated as decrease in nitrogen, mg/cc in 24 hours.

Citrate buffer pH 2.6, acetate pH 3.6 and 4.2, phosphate pH 5.5, 6.1 and 7.4. Concentration of cysteine and glucose respectively 0.3 M. and 0.5 M.

In contrast to the other amino acids the cysteine amino group-aldehyde reaction was demonstrable in acid solutions. Figure 3 shows the rate of reaction (disappearance of VAN SLYKE nitrogen) in 0.2 M buffer solutions between pH 2.6 and 7.2. The optimal pH of the reaction was not in acid solutions. The spontaneous oxidation of cysteine by air starts at pH 8 and increases with the rise in pH. Accordingly the cysteine-glucose reactions were studied at pH 7.4.

In control solutions with cysteine alone the cysteine concentration had not changed after 48 hours. The stability of the cysteine-sugar product was rather strong in acid solutions. Shaking for 30 minutes in the VAN SLYKE determination did not split the product. When mixed with HCl to a final concentration of 10 per cent the cysteine-sugar product was not split after remaining 8 hours at room temperature. Heating in a sealed tube in water bath for 30 minutes in 10 per cent HCl hydrolysed 10 per cent of the compound.

A series of equimolecular cysteine-glucose solutions (0.3 M) were constantly kept at pH 7.4 and 40° 10 N NaOH being added from a micro burett while N₂ was bubbling through the solution. Parallel determination of VAN SLYKE nitrogen, acetone titrations on amino nitrogen and cysteine-cystine determinations were made. The results are given in table 5.

Table 5.

Parallel determinations of van Slyke nitrogen, titrable amino groups, cysteine and cystine in cysteine-glucose solution incubated at 40° C and pH 7.4.

Time in hours	Van Slyke nitrogen in mg/cc	cc n/40 HCl required in titration of 75 c. mm. of solution	c. mm. 10 NaOH added per cc solution to bring pH to 7.4	Cysteine in mg/cc	Cystine in mg/cc	cc n/40 NaOH added from time 0 calc. on 75 c. mm. solution	Column 3 — blank values — column 7 = titrable amino groups
1	2	3	4	5	6	7	8
0	3.95	1.32	0	27.5	0	0	0.69
1	3.18	1.38	1.95	28.0	0	0.060	0.69
2	2.76	1.41	1.67	28.0	0	0.112	0.67
4	2.55	1.43	2.31	27.5	0	0.180	0.64
6	2.00	1.48	2.00	23.5	0	0.228	0.63
6	1.75	1.57	1.83	21.1	0	0.284	0.66
25	1.00	1.60	2.00	13.5	0	0.344	0.63

Concentration of cysteine HCl and sugar respectively 0.3 M. and 0.5 M.

It is made clear in column 8 (the titration values corrected for blank values by the titration and the added amounts of NaOH) that the nitrogen atom in the compound formed can still be titrated as amino nitrogen in spite of the apparently decreased basic properties of the cysteine-sugar compound. In all prob-

ability this must contain the sulphur atom in such a position as to be underterminable either as cysteine or cystine by the method here used. These conclusions are drawn from the corresponding amounts of amino nitrogen and cysteine left after the reaction has ceased.

Various efforts were made to isolate the cysteine-sugar compound. In this connection may be mentioned that SCHUBERT (1936) studied reactions between organic sulphur and aldehyde compounds. Fractionation with alcohol, phenol and pyridine gave inhomogeneous products. There seemed to be a tendency in alcohol-water solutions of this product to dissociate into sugar and cystine. Solutions containing the cysteine-sugar product were aerated with traces of FeCl_3 to remove cysteine which had not reacted with sugar. Then the cysteine-sugar compound was precipitated with CuAc_2 and ethyl alcohol to 60 per cent. The precipitate was treated with H_2S which was removed with bubbling N_2 . Through this oxidation and reduction a product was obtained containing no free VAN SLYKE nitrogen but with sulphur clearly determinable as cysteine-sulphur according to KASSEL and BRAND (1938). A similar type of substance was obtained directly when cysteine reacted with methylglyoxal in equimolar amounts (table 4). It is of special interest to note that the thiol test with sodium nitroprusside gave a negative result on the same conditions. While it was possible to isolate this lateral reaction with a free thiol group, the sulphur in the main reaction was fixed in a position of another type, where it could not be determined as thiol or S—S sulphur.

The method finally adopted to isolate the cysteine-glucose compound was as follows. 30 mM each of cysteine and glucose were solved in 50 cc glass distilled water at pH 7.4 and 50 mg. diethyl-dithio-carbamide added. After gentle shaking the diethyl-dithio-carbamide-copper was extracted in five minutes with amyl alcohol. The water solution was kept at 40° C and pH 7.4 as described above. The reaction usually ceased after 12 hours (fig. 2) and the solution was concentrated in vacuum to a sirup and precipitated with water-free alcohol 10—15 volumes. The mixture was kept over night at 0°, centrifuged and the precipitate dried with alcohol and ether. 2 g. of the precipitate was thoroughly extracted with 20 cc of concentrated acetic acid. About 50 per cent of the material was solved in the acetic acid, and the solution was precipitated with ether. Both fractions

were dried with alcohol and ether. Analysis of the soluble and insoluble acetic acid fractions are given in table 6. Sulphur was determined with the Pregl method.

Table 6.

Analysis of cysteine-glucose compound.

S a m p l e	Total nitrogen	Sulphur	Van Slyke nitrogen	Cysteine + cystine	Sugar	Yield
Calculated for	per cent	per cent	per cent	per cent	per cent	per cent
$C_9H_{17}O_7NS$	4.9	11.3				
Insoluble in acetic acid	4.8	11.2	0	0	3	48
Soluble in acetic acid .	2.2	5.8	1.8	15	25	

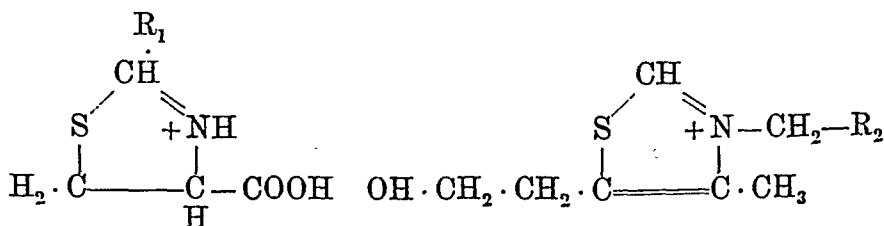
The acetic acid extraction removed small quantities of sugar and cysteine, which had not reacted, together with a part of the final product. The rest of the material was obtained in a practically pure form. It was easily soluble in water. When exposed to air it absorbed water and it was necessary for the sake of analysis to dry the product to constant weight over P_2O_5 . The constitutional similarity with the thiazol compounds (see discussion) made it desirable to test the effect of oxidation reactions on the compound. Weak oxidants, as the oxygen of air, had no activity even in the presence of copper. Hydrogen peroxide easily split the product into cystine and sugar. The same effect was obtained by dehydration. Methylenblue was reduced and free cystine and glucose isolated. The ultimate biological effect was tested with the phycomyces method. This test was performed by dr NIELSEN of the Biological Department of Carlsberg Laboratory. The thiazol derivative did not increase the activating influence of the pyridine part of vitamin B_1 . It is quite clear that the thiazol compound could not be coupled directly in its isolated form.

Discussion.

The investigation of the reaction between aldehyde groups and amino acids was undertaken with a view to isolating some of the reaction products for a further study of the reactivity of the linkage between the amino and aldehyde groups. The ten-

dency to form bonds at the physiological hydrogen concentration is very weak in all amino acids with the exception of cysteine. In this case the aldehyde group is linked to two groups in the molecule of the amino acid, the thiol and amino group. This gives strength to the compound. A comparison of the reactivity of amino acids and dipeptides proved that slight increases in the dissociation of the amino groups did not facilitate the reaction. From a structural point of view it may be mentioned that there was no difference in the reactivity of the natural and unnatural amino acids. Experiments later to be published confirm the view that the undissociated amino groups are the most reactive. The activating influence of phosphate remains to be explained. Several possibilities offer themselves. The phosphate ions may act directly on the aldehyde-amino group association; it is known that metaphosphoric acid derivatives form compounds with amino groups. It has also been demonstrated at Warburgs laboratory that the phosphoric acid forms compounds with the carboxylic groups in intermediately formed carbohydrate compounds of the fermentation reaction. Experiments now in progress seem to show that the reactivity of alanine with glucose is stimulated by the estrification of the carboxylic group. It is possible that the phosphate ions may act in some similar way.

Interest was concentrated on the cysteine sugar products. It is possible to prove that complete reaction depends on the forming of a second bond to the thiol group. A comparison between the rate of disappearance of VAN SLYKE nitrogen and thiol group showed that the initial reaction included the forming of the bond between the amino and aldehyde groups. The properties of the finally isolated compound corresponded to a thiazol compound. Structural similarities with the thiazol part of the vitamin B₁ made a testing of the biological properties desirable. Obviously the structural differences could not be eliminated by the synthetical activity of the phycomyces bacterias.



The cysteine-glucose compound. The thiazole half of vitamin B₁.

Several efforts were made to oxidize or dehydrate the isolated compound but the result was unexceptionally an opening of the thiazol ring. It is possible that by methylation of the amino group, decarboxylation or estrification of the carboxylic group and by replacing the glucose radical with some phospho-glycerinaldehyde compound a more active thiazol compound can be obtained. From a biological point of view this reaction between sulphur containing amino acids and aldehydes may be of further interest. Anyhow the occurrence of considerable amounts of cysteine in the liver extracts may explain some of the rapid and complete disappearances of VAN SLYKE nitrogen in these extracts (ÅGREN 1940).

Summary.

1. The reaction between glucose and the usually occurring amino acids and some peptides has been studied at pH 7.4.
2. Only with cysteine an approach to a complete reaction was obtained.
3. A cysteine-sugar compound with thiazol structure has been isolated.
4. The reaction between amino acids and glucose is accelerated in the presence of phosphate.

The author wishes to express his gratitude to Professor K. LINDERSTRÖM-LANG for extending the facilities of the Carlsberg Laboratory and for his untiring interest in the work. This has been supported by a grant from the Therese and Johan Anderssons Minne Foundation.

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(From the Biochemical Department, Karolinska Institutet, Stockholm.)

On the Peptidase Activity in the Cattle Muscle.¹

By

GUNNAR ÅGREN.²

To facilitate an examination of transamination from peptides in cattle muscle, it was desirable to separate the reaction from the interfering influence of eventually contaminating peptidase activity. The present paper is confined to a study of peptidase activity in the cattle muscle. The experiments were extended in two directions. To begin with, an effort was made to suppress the peptidase activity by means of the usual enzyme inhibitors. When this method proved unsatisfactory several peptides were investigated with regard to their capacity to resist enzymatical digestion. Under the conditions maintained by BRAUNSTEIN and KRITZMANN (1937) in their study of transamination, glycyl-aminobenzoic acid and valyl-glycine was not split.

Experimental Procedures.

Cattle diaphragm muscle was chilled in the slaughter-house immediately after the death of the animal, and cut very fine in a mincer. The minced muscle was suspended in 4 parts of 0.125 per cent KHCO_3 . A part of the solution was directly taken to peptidase analysis and the rest was incubated in THUNBERG tubes at 40° , and shaken for 30 minutes for a new test. Within this space of time the transamination would have ceased. There was a possibility that the peptidases in the muscle would be more completely extracted during the shaking period. After the digestion there followed a titration of the newly formed amino and carboxylic groups with the micro methods of LINDERSTRÖM-LANG and HOL-

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² Fellow of the Rockefeller Foundation 1938--1939.

TER. The time for digestion was limited to 30 minutes according to reasons stated above. Glutamic acid was determined by the JONES and MOELLER method (1928) in the modification of BRAUNSTEIN and KRITZMANN (1937).

Results.

Alanyl-glycine (AG) and alanyl-glycyl-glycine (AGG) were easily split by peptidases in the muscle extract as seen in table 1.

Table 1.

Digestion of alanyl-glycine (AG) and alanyl-glycyl-glycine (AGG) in fresh and incubated muscle.

	Fresh muscle +		Incubated muscle +	
	AG	AGG	AG	AGG
Digestion in c.mm.	3.2	11.4	5.9	12.5

Concentration of peptide solutions 0.2 M. Digestion with 7 c.mm. of substrate and 7 c.mm. of filtered muscle extract. Digestion values in c.mm. $n/20$ HCl in 90 per cent alcohol. Incubation time 30 minutes. Temperature 40° .

Muscle tissue evidently contained considerable amounts of dipeptidase and aminopolypeptidase. The enzyme concentrations were increased by the incubation process. The undesirable effect of the peptidase activity was clearly demonstrable in a transamination experiment with alanyl-glycine, the experimental conditions being the same as given by BRAUNSTEIN and KRITZMANN (1937). Table 2.

Table 2.

Digestion of alanyl-glycine in muscle extract.

S a m p l e	Incubation time in minutes	Total van Slyke nitrogen in mg/cc.	Van Slyke nitrogen of the glutamic acid fraction in mg/cc.
Alanyl-glycine	0	0.76	0.38
	30	0.98	0.83
Control	0	0.19	0.24
	30	0.19	0.22

3 g. of muscle + 1.2 cc of 2 per cent KHCO_3 + 3 cc (90 mg) of a d,l-alanyl-glycine solution + 8 cc of water. Control without alanyl-glycine. Incubation at 40° .

The difference in van Slyke nitrogen between the alanyl-glycine solution before incubation, and the control solution, corresponds to an almost complete digestion of the added alanyl-glycine in 30 minutes. The increase in glutamic acid van Slyke nitrogen depends on the presence of glycine, liberated during the digestion.

Next an effort was made to suppress the peptidase activity by enzyme inhibitors. The choice of inhibitor was to some extent free, since BRAUNSTEIN and KRITZMANN (1937) had demonstrated that transamination proceeded rather independantly of oxygen or nitrogen atmosphere. Some of the results obtained with enzyme inhibitors are given in table 3.

Table 3.

Influence of enzyme inhibitors on the muscle di-peptidases.

Peptide added	Inhibitor	Digestion in c.mm.
Alanyl-glycine 0.2 M.	—	8.3
Alanyl-glycine 0.2 M.	Bromoacetate 1:5000	8.2
Alanyl-glycine 0.2 M.	Sodium arsenite m/100	8.2
Alanyl-glycine 0.2 M.	Potassium cyanide m/100	5.8
Alanyl-glycine 0.2 M.	Sodium fluoride m/100	8.1
Leucyl-glycine 0.2 M.	—	2.2
Leucyl-glycine 0.2 M.	Sodium cyanide m/100	2.3

Muscle extract incubated 30 minutes at 40°, filtered. Digestion with 7 c.mm. of substrate + 7 c.mm. of muscle extract. Digestion as c.mm. n/20 HCl in 90 per cent alcohol.

The results with the enzyme inhibitors were not promising, as a cause of which interest turned to the substrates. Peptides were tested regarding their capacity to resist enzymatical digestion. Glycyl-aminobenzoic acid is known to be slowly digested by glycerine extracts of the intestinal mucous membrane. The peptide was synthesized according to one of FISCHER's methods (1905), the ortho-derivate being preferable to the para-compounds for enzymatical analysis, as it was more easily soluble.

Solubility of glycyl-aminobenzoic acid compounds.

Solubility in mg/cc at 20°.

Ortho-derivate	11 mg
Para-derivate	5.5 mg
Meta-derivate	13 mg

In the enzymatic experiments the solution of the ortho-compound was saturated at 40°, the concentration being about 0.1 M (19 mg/cc). Still the solubility of glycyl-aminobenzoic acid was so low, that in functioning as amino group donator, the effect was found to be just within the range of the analytical methods used in the transamination experiments. A few other peptides were included in the enzymatical analysis: Leucyl-glycyl-glycine, leucyl-phenyl-amino-acetic acid and valyl-glycine. Valyl-glycine was synthesized according to FISCHER (1907). The results of the digestion with muscle peptidases are listed in table 4. The bicarbonate suspension of muscle was incubated at 40° for 30 minutes, filtered and used as enzyme solution.

Table 4.

Peptide digestion in muscle extract.

Peptide added	Digestion in c.mm.	Substrate + en- zyme solution in c.mm.
Alanyl-glycine 0.2 M.	8.8	7 + 7
Glycyl-aminobenzoic acid 0.1 M.	0	7 + 7
Leucyl-glycyl-glycine 0.2 M.	1.8	7 + 7
Leucyl-glycyl-glycine 0.01 M.	1.6	70 + 7
Leucyl phenylaminoacetic acid 0.05 M.	0.6	30 + 7
Valyl-glycine 0.2 M.	0.2	7 + 7

The enzyme-substrate solutions incubated 30 minutes at 40°. Digestion in c.mm. n/20 HCl in 90 per cent alcohol. Glycyl-aminobenzoic acid followed by titrations on the carboxylic groups with n/20 (CH₃)₄NOH.

The only two peptides which could be used in transamination were apparently glycyl-aminobenzoic acid and valyl-glycine. Test experiments were made with the standard concentration of amino group donators (100 μ Mol/g of muscle) in the muscle-brei using the two peptides as donators. An increase of van Slyke nitrogen

indicating a hydrolysatation could not be proved after 30 minutes' incubation at 40°, as seen in table 5.

Table 5.
Digestion of peptides in muscle extracts.

Peptide added	Incubation time in minutes	Van Slyke nitrogen in mg/cc.
alanyl-glycine	0	0.73
	30	0.96
valyl-glycine	0	0.73
	30	0.72
glycyl-aminobenzoic acid	0	0.76
	30	0.77

3 g. of muscle + 1.2 cc. of 2 per cent KHCO_3 + peptide solution (100 μ Mol/g muscle) + water to fill up to 12 cc. Shaking at 40° in water bath.

Discussion.

The peptidase activity in cattle muscle is about half as high as in the intestinal mucousa of hog, when the digestion of alanyl-glycine is taken as a reference. Especially di- and tripeptides containing alanine are especially sensitive to attack. Replacing alanine in alanyl-glycine with leucine or valine serves as an effective protection for the peptide against digestion. It was of interest to note that the splitting of alanyl-glycine was to some extent inhibited in the presence of cyanide, while the digestion of leucyl-peptides was not influenced. It is a well known fact that the affinities of other enzyme systems, e. g. lactic dehydrogenase, are not strictly limited to one substrate. Homologous compounds may also react, but the rate of reaction rapidly falls off as the carbon chain is lengthened. Following the hint, it was possible to demonstrate that glycyl-aminobenzoic acid and valyl-glycine could be suitable amino group donators in transamination experiments, as they were not split by peptidases in the short time taken for the experiment.

Summary.

Cattle diaphragm muscle contains di-peptidases and aminopoly-peptidases, splitting alanyl-peptides especially. Valyl-glycine

and glycyl-aminobenzoic acid are not hydrolized in experiments of short durability. The digestion of alanyl-peptides is partially inhibited by cyanide.

The author wishes to express his gratitude to Professor K. LINDERSTRÖM-LANG for extending the facilities of the Carlsberg Laboratory, and for his untiring interest in the work and valuable criticism of the manuscript.

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Die Stabilität der organischen Phosphorverbindungen und Phosphatase in Pferdeblut bei dessen Aufbewahrung in vitro.¹

Von

KNUT SJÖBERG.

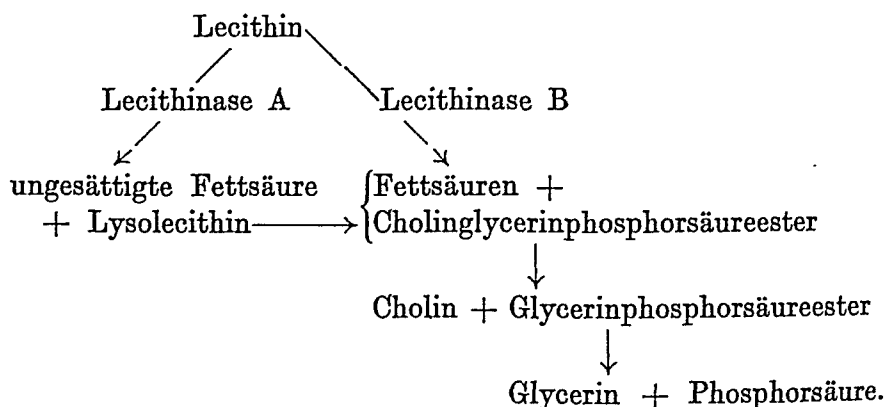
Es ist eine alte Erfahrung, dass eine Hydrolysierung der Phosphorsäureester in hämolysiertem oder mit Kohlensäure gesättigtem Blut stattfindet. Es finden sich jedoch keine eingehenderen Untersuchungen über die Stabilität des Blutes, speziell der Phosphorverbindungen, in vitro vor. Nachdem FÄHRÆUS (1939) und BERGENHEM (1939) gezeigt haben, dass die Erythrozyten, besonders im Pferdeblut, aus der Münzform in eine mehr sphärische Form übergehen, falls das Blut bei 38—40° C aufbewahrt wird, drängt sich die Frage auf, ob diese Veränderung mit einigen bedeutenderen chemischen Umwandlungen vereinigt ist. Nach der Ansicht der genannten Forscher beruht die Veränderung der Form der Blutkörperchen auf der Bildung von Lysolecithin im Plasma, einem Stoff, der die Blutkörpermembran beeinflusst. Die Permeabilität derselben nimmt zu, und Wasser nebst einem Teil gelöster Stoffe diffundiert aus dem Plasma in die Erythrozyten und eventuell auch in entgegengesetzter Richtung. Es lässt sich denken, dass schon dieses Verhalten eine solche Änderung des Milieus zu bedingen vermag, dass die Phosphoresterasen ihre hydrolysierende Wirkung ausüben können.

Die wichtigsten Phosphorsäureester in den Erythrozyten bestehen aus Hexosemonophosphorsäureestern (KAY und ROBISON, 1924), Glycerinsäurediphosphorsäureestern (JOST 1927) und Adenylpyrophosphat (Adenosintriphosphorsäure) (LOH-

¹ Der Redaktion am 1. Juli 1940 zugegangen.

MANN, 1928, 1929). Dazu kommen die phosphorhaltigen Lipide vom Lecithintypus, die hier der Kürze halber mit Lecithin bezeichnet werden. Alle diese Phosphorverbindungen können von verschiedenen Enzymen gespalten werden, von denen im Blut nur die Monophosphoesterase eingehender untersucht wurde. Pyrophosphatase ist im Blut von KAY (1928) und von ROCHE (1931) nachgewiesen worden, wurde aber keinen eingehenderen Untersuchungen unterzogen. Ich vermochte jedoch neulich nachzuweisen, dass dieses Enzym hauptsächlich in den Erythrozyten vorkommt, und dass seine Menge bei verschiedenen Tierarten variiert (noch nicht veröffentlicht).

Das Lecithin kann nach BELFANTI, CONTARDI und ERCOLI (1936) auf zweierlei Art nach folgendem Schema gespalten werden:



Die primär gebildeten Stoffe werden weiter von anderen Enzymen zerteilt, so dass schliesslich anorganische Orthophosphate freiwerden. Das Vorkommen von Lecithinase A im Blut ist durch BERGENHEMS (1939) Untersuchungen als sichergestellt zu betrachten. Inwiefern sich Lecithinase B und diejenigen Enzyme, welche weiter die Spaltungsprodukte hydrolysieren, im Blut vorfinden, scheint nicht untersucht worden zu sein.

Der Zweck der vorliegenden Arbeit lag in der Untersuchung des Verlaufes der Spaltung der Phosphorsäureester im Pferdeblut, das teils bei Zimmertemperatur (etwa 17° C) aufbewahrt, teils auf Körpertemperatur (37°) erwärmt und gehalten wurde.

Die Untersuchung ergab, dass die Phosphorsäureester bei Zimmertemperatur bedeutend langsamer hydrolysiert werden als bei Körpertemperatur, ein Verhalten, das ja mit Rücksicht auf die Abhängigkeit der enzymatischen Reaktionen von der Temperatur zu erwarten war. Bei Zimmertemperatur zeigten die Blut-

proben während der ersten 24 Stunden nur eine unbedeutende Spaltung. Auch das nach der Hämatokritmethode bestimmte Volumen der Blutkörperchen änderte sich nicht. Allmählich begannen die Blutproben zu hämolysieren, und im Zusammenhang hiermit trat eine Hydrolyse der Phosphorsäureester ein, deren Schnelligkeit mit fortdauerndem Blutkörperzerfall zunahm.

In denjenigen Fällen, in welchen das Blut bei 37° C aufbewahrt wurde, änderte sich die Form der Blutkörperchen in 24 Stunden in Richtung einer Sphärozytose. Im Zusammenhang hiermit stiegen die Hämatokritwerte. Setzt man voraus, dass die Oberfläche der Blutkörpermembran unverändert ist, und berechnet man den Grad der Änderung des Volumens, wenn die Form aus der Münzform in die sphärische übergeht, so ergibt sich eine Änderung von 1 : 1.75. In vorliegenden Versuchen änderten sich die Hämatokritwerte nach 24 Stunden durchschnittlich im Verhältnis von 1 : 1.6, also zu einem etwas niedrigeren Wert als dem berechneten. Es ist jedoch zu beachten, dass nicht alle Blutkörperchen vor und nach der Erwärmung gerade die ideale Münzen- bzw. Sphärenform besitzen. Ausserdem war zuweilen eine schwache Hämolysen inzwischen eingetreten, was eine Verminderung des Hämatokritwertes bedingt.

Zur Untersuchung einer eventuell gesteigerten Permeabilität im Zusammenhang mit der Veränderung der Blutkörperchen wurde die Menge gewisser anorganischer Ionen im Plasma vor und nach der Erwärmung bestimmt. Es zeigte sich, dass Na⁺, Ca⁺⁺ und Cl⁻, aus dem Plasma zu den Erythrozyten in derselben Proportion wie das Wasser wanderten, weshalb die Blutkörpermembran für diese Ionen vollständig permeabel worden war, K⁺ wanderte in entgegengesetzter Richtung von den Erythrozyten zum Plasma, was auf dem grösseren Gehalt der ersteren an dieser Ionenart beruht. In den bei 17° aufbewahrten Blutproben geschah während der ersten 24 Stunden kein Austausch von Ionen zwischen Plasma und Blutkörperchen.

Die Untersuchung der Resistenz der Blutkörperchen in NaCl-Lösungen von wechselnder Konzentration zeigte eine Verminderung der Resistenz.

In den meisten Fällen liess sich nach 24 Stunden bei 37° keine Hämolysen feststellen, nach 48 Stunden fand sich jedoch eine sehr kräftige oder vollständige Auflösung der Blutkörperchen vor. Auch in denjenigen Fällen, in welchen keine Hämolysen stattgefunden hatte, begannen die Phosphorsäureester zu zerfallen,

und nach 2—3 Tagen war die ganze Menge sog. säurelöslichen Phosphors in anorganisch gebundenen Phosphor übergegangen. Dieses zeigt, dass das Blut alle die Typen von Phosphatasen enthält, welche die Phosphorsäureester hydrolysieren. In gewissen Fällen erhielt ich mehr anorganischen Phosphor als es dem ursprünglichen säurelöslichen Phosphor entsprach. Der Überschuss muss vom Lecithin herkommen, das also einer Zerteilung nach dem genannten Schema anheimfällt, wenngleich diese Hydrolyse verhältnismässig langsam verläuft.

Die Phosphormonoesterase kommt beim Pferde sowohl im Plasma als auch in den Erythrozyten vor. In den hier untersuchten Fällen variierte die in BODANSKY-Einheiten (B. E.) ausgedrückte Phosphatase im Plasma zwischen 2.1 und 5.1, durchschnittl. 3.1, und in den Erythrozyten zwischen 9.5 und 16.4, durchschnittl. 12.0, alles pro 100 ml berechnet. Der Gehalt an Phosphormonoesterase war also in den Erythrozyten ungefähr 4mal höher als im Plasma.

Die Pyrophosphatasen kommen ebenfalls in bedeutend grösserer Menge in den Erythrozyten als im Plasma vor. Das Verhältnis ist nach unveröffentlichten Untersuchungen im Durchschnitt 24 : 1.

Da die Phosphorsäureester in den Erythrozyten vorkommen, muss die Spaltung entweder daselbst oder auch im Plasma vor sich gehen, nachdem die Ester in dasselbe hindurchdiffundieren. Dieses kann jedoch erst nach einer genügenden Zunahme der Permeabilität der Blutkörpermembran stattfinden.

Der Umstand, dass eine kräftige Phosphatasewirkung auch bei 17° in hämolysiertem Blut eintreten kann, scheint darauf hinzuweisen, dass eine Aktivierung der Enzymtätigkeit bei der Hämolysen stattfindet. Die Bestimmung der Phosphatasewirkung im Blut geschieht in der Regel in hämolysierten Proben. In unveränderten Blutproben können sich die Enzyme in einem solchen Zustand befinden, dass sie keine Wirkung ausüben. Man weiss aus anderen Fällen, dass das Enzym erst in eine aktive Form übergeführt werden muss.

Zwecks Untersuchung des Einflusses der Hämolysen wurde die Phosphormonoesterase nach BODANSKY (1933) in einer mit dem Blut isotonischen Reaktionsmischung bestimmt. Obgleich die Erythrozyten nicht der Hämolysen anheimfielen, erhielt ich für die Phosphatasewirkung denselben Wert wie bei der gewöhnlichen Bestimmung in hämolysierten Proben. Die Phosphormonoeste-

rase scheint sich an der Oberfläche des Blutkörperchens zu befinden, wo sie die Glycerinphosphorsäure in der Lösung hydrolysieren kann, aber nicht die Phosphorsäureester in den Blutkörperchen.

Diese Resultate deuten darauf hin, dass sich die Phosphormonoesterase und die Phosphorsäureester in den Blutkörperchen in einem solchen Zustand befinden, dass die ersteren nicht die letzteren anzugreifen vermögen. Erst nachdem die Blutkörperchen gewissen Veränderungen anheimgefallen sind, beginnt das Enzym seine Tätigkeit zu entfalten. Eventuell kann hierbei irgendein Aktivator beteiligt sein.

In künstlich hämolysierten Blutproben könnte man auf Grund der früheren Hinweise eine relativ schnelle Hydrolyse der eigenen Phosphorsäureester der Blutkörperchen auch bei Zimmertemperatur erwarten. In einem Versuch, in dem eine Hämolysse durch Zusatz von Saponin bewirkt worden war, erhielt ich auch eine unmittelbar eintretende Hydrolyse. Schon nach 3 Stunden bei 17° war die Spaltung deutlich, während sich in den bei 37° aufbewahrten nicht mit Saponin versetzten Proben nach 7 Stunden in der Regel keine Veränderungen nachweisen liess. In diesen letzteren Proben war die Zunahme der Permeabilität der Blutkörpermembran während dieser Zeit offenbar noch nicht so weit vorgeschritten, dass eine vollständige Kommunikation zwischen Erythrozyten und Plasma erreicht wurde.

Diese Verhältnisse gelten also für die Phosphormonoesterase. Das Verhalten der Pyrophosphatase unterliegt einer Untersuchung und wird in einer späteren Mitteilung der Diskussion unterzogen werden.

Die enzymatische Spaltung des Lecithins wird durch die Bestimmung des Lipoidphosphorgehalts verfolgt. Hierbei erhält man nicht etwa einen Ausdruck für die Wirkung der Lecithinase A, also die Überführung des Lecithins in Lysolecithin, sondern für die Wirkung der Lecithinase B, die Abspaltung der beiden Fettsäuremoleküle. Gleichzeitig folgte ich in gewissen Fällen der Steigerung Alkohol-Äther-löslicher Fettsäuren. Diese brauchen jedoch nicht allein vom Lecithin herzustammen, sondern können auch durch Zerteilung von Neutralfett und Cholesterinestern gebildet werden.

Der Lipoidphosphorgehalt sank nach 24 Stunden langer Erwärmung bei 37° in einigen Versuchen im Plasma und auch in den Erythrozyten. Im Zusammenhang mit der eintretenden Hämolyse

lyse wurde die Spaltungsschnelligkeit grösser und die Lecithinmenge sank kräftig in beiden Medien.

Die Mengen freier Alkohol-Ather-löslicher Fettsäuren stieg sowohl in den Erythrozyten als auch im Plasma.

Demnach kommen im Blut auch Lecithinase B und die Diesterase vor, die Cholinglyzerinphosphorsäureester spaltet.

Experimenteller Teil.

Die Versuche wurden mit Pferdeblut ausgeführt. Die Bestimmung des Phosphors geschah nach FISKE und SUBBAROW (1925) mit Hilfe des photoelektrischen Kolorimeters.

Zur Bestimmung des Lecithins wurden die Blutproben nach THEORELL (1930) extrahiert und danach der Phosphorgehalt auf die gewöhnliche Art festgestellt.

Die übrigen Blutbestandteile wurden nach den gebräuchlichen Methoden analysiert. Die Analysen wurden sowohl mit Totalblut als auch mit Plasma ausgeführt, und nach der Bestimmung der Hämatokritwerte berechnete ich die Konzentrationen in den Blutkörperchen.

Die Aufbewahrung der Blutproben geschah einen bis mehrere Tage nach Zusatz von Na-Zitrat oder Heparin zur Verhinderung der Koagulation teils bei 37° C und teils bei Zimmertemperatur (17°).

Das Blutkörpervolumen veränderte sich während 48 Stunden nicht in denjenigen Blutproben, welche bei der niedrigeren Temperatur aufbewahrt wurden. Bei längerer Aufbewahrung trat Hämolyse ein, und dabei kam natürlich eine Verminderung der Hämatokritwerte zustande.

Figur 1 zeigt die Zunahme der Hämatokritwerte in sechs Blutproben, die bei der höheren Temperatur aufbewahrt wurden. Der linke Pfeiler in jeder Gruppe gibt den Wert unmittelbar nach der Blutprobenentnahme an und der rechte nach 24 Stunden langer Aufbewahrung der Probe bei 37°. In mehreren Fällen war während dieser Zeit bereits ein gewisser Grad von Hämolyse eingetreten, was natürlich eine Senkung des Hämatokritwertes herbeiführt. Bei weiterer Aufbewahrung stieg der Hämolysegrad schnell, und nach 2—3 Tagen war die Hämolyse vollständig.

Das Blutkörpervolumen nahm während der ersten 24 Stunden durchschnittlich im Verhältnis 1 : 1.6 zu, also bis zu einem etwas niedrigeren als dem berechneten Werte 1 : 1.75.

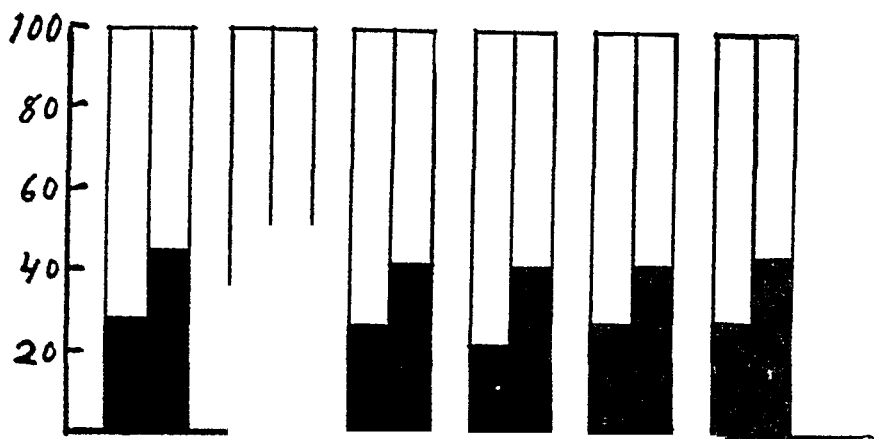


Fig. 1. Hämatokritwerte von 6 Pferdeblutproben. Linker Pfeiler vor der Erwärmung. Rechter Pfeiler nach 24 Stunden bei 37°.

Es lässt sich denken, dass die Ursache der Diffusion der Flüssigkeit in das Blutkörperchen hinein in einer Steigerung des osmotischen Druckes auf Grund eines Freiwerdens von Ionen liegt. Es ist nicht möglich, den osmotischen Druck direkt im Blutkörperchen festzustellen. Die Bestimmung des Gefrierpunktes des Plasmas vor und nach der Erwärmung ergab, praktisch genommen, denselben Wert, weshalb der osmotische Druck im Plasma jedenfalls keiner Veränderung anheimgefallen war.

Eine wahrscheinlichere Erklärung der Diffusion ist eine Veränderung der Permeabilität der Blutkörpermembran. Zur Untersuchung, ob eine solche Veränderung tatsächlich stattgefunden hatte, wurde teils die Resistenz der Blutkörperchen in Kochsalzlösungen verschiedener Konzentration, teils der K-, Na-, Ca- und Cl-Gehalt im Plasma vor und nach der Erwärmung bestimmt.

Vor der Behandlung fand sich der Beginn schwacher Hämolyse in 0.55-proz. NaCl-Lösung, und vollständige Hämolyse trat in 0.4-proz. Lösung ein. Nach der Behandlung waren die entsprechenden Ziffern 0.75 und 0.6. Die Resistenz war somit gesunken.

Die Resultate der Analysen der obengenannten Ionen finden sich in Figur 2. Wie vorher erwähnt, wurde in der Regel Natriumzitat zugesetzt. Dieses bewirkte eine Steigerung des Na-Gehalts und einen Eingriff in das osmotische Gleichgewicht zwischen Plasma und Erythrozyten. Trotzdem liess sich in den Kontrollproben bei 17° keine Diffusion von Na-Ionen oder anderen Ionen feststellen. Figur 2 zeigt jedoch, dass in den erwärmten Proben

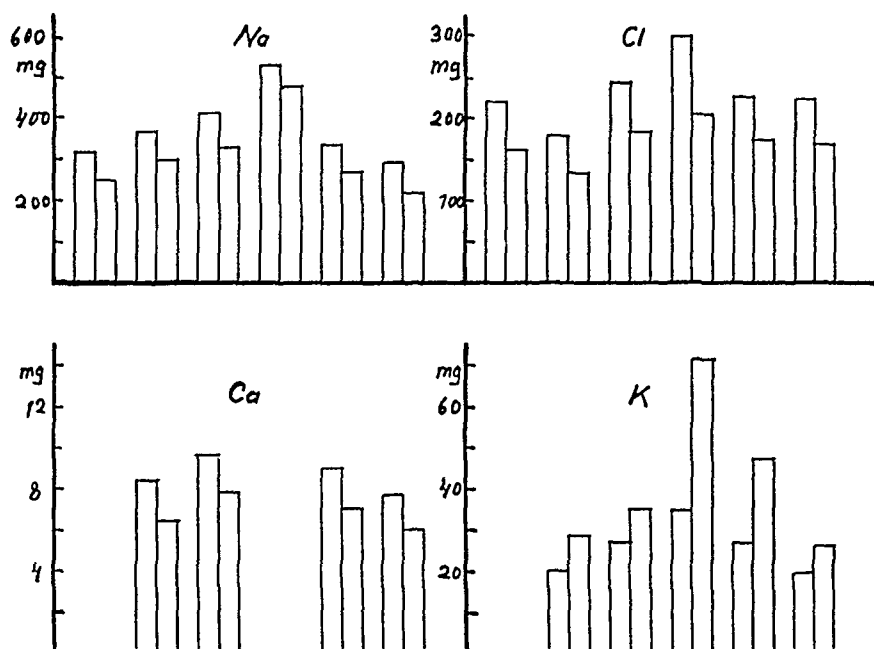


Fig. 2. Die Pfeiler geben die verschiedenen Stoffe in mg im Plasma von 100 ml Blut vor der Erwärmung und nach 24 Stunden langer Erwärmung auf 37° an.

die genannten Ionen durch die Blutkörpermembran wanderten, Na^+ , Ca^{++} und Cl^- in Richtung vom Plasma nach den Blutkörperchen, K^+ in entgegengesetzter Richtung. Dieses hängt mit den ursprünglichen Konzentrationen dieser Ionen zusammen, nur K^+ kommt im Überschuss in den Erythrozyten vor. Die Verminderung des Gehalts an Na^+ , Ca^{++} und Cl^- ist im Durchschnitt proportional der diffundierenden Wassermenge, was also zeigt, dass die Blutkörpermembran für diese Ionen völlig permeabel geworden war.

Betreffs der Phosphationen stellt sich das Verhalten komplizierter, da man hier nicht nur mit einer Diffusion zu tun hat, sondern die Hydrolyse der Phosphorsäureester vorherrscht. Tabelle 1 zeigt einige Beispiele für die Veränderungen der verschiedenen Phosphorverbindungen. Est. P bezeichnet denjenigen Phosphor, welcher als organischen Phosphorsäureester gebunden ist (Leithin-P nicht mitgerechnet).

Bei 17° findet eine langsame Zerteilung der Phosphorsäureester während der beiden ersten Tage statt. Dieses beeinflusst die Menge an anorganischem P im Plasma nur unbedeutend, da die Permeabilität für Phosphationen nur gering ist. Nach 2—3

Tabelle 1.

Die Verteilung der Phosphorverbindungen und der Phosphormonoesterase zwischen dem Plasma und den Erythrozyten im Pferdeblut unter verschiedenen Bedingungen in vitro. Die Werte geben die Mengen im Plasma und Erythrozyten vom 100 ml Blut in mg an.

Nr.		Blutkörp. vol.	Plasma			Erythrozyten			Bemerkungen
			anorg. P mg	Est. P mg	Phos- phata- se B.F.	anorg. P mg	Est. P mg	Phos- phata- se B.E.	
1a	0	28	2.53	0	1.89	0.28	13.45	4.58	17°
	48	28	2.53	0	—	0.46	13.26	—	
	72	21	1.98	6.74	4.91	0.64	5.89	1.31	Hämolyse
	96	20	5.45	6.50	5.95	1.05	3.25	0.19	Kräftige Hämolyse
1b	0	28	2.53	0	1.89	0.28	13.45	4.58	37°
	7	—	2.53	0	—	—	—	—	
	24	45	9.82	—	—	0.60	5.73	—	
	48	25	12.19	0.50	2.24	1.01	2.55	2.08	Hämolyse
	72	0	14.43	1.80	4.47	—	—	—	Vollständige Hämolyse
2a	0	45	1.76	0	1.16	0.64	15.69	4.28	17°
	24	47	2.05	0.82	1.16	1.45	13.77	3.78	
	48	47	2.58	0.28	1.15	3.29	11.94	3.38	
	72	48	2.66	0.90	2.02	6.24	7.37	2.11	Ein wenig Hämolyse
2b	0	45	1.76	0	1.16	0.64	15.69	4.28	37°
	24	69	4.60	0.42	1.34	6.44	6.63	2.36	
	48	57	8.17	0.26	3.71	8.13	1.53	0	Hämolyse
2c	0	0	2.15	14.08	6.35	—	—	—	20 mg Saponin pr 100
	4	0	3.06	13.17	—	—	—	—	ml. 17°. Vollständige
	24	0	5.34	10.89	6.26	—	—	—	Hämolyse.
	48	0	6.40	9.83	4.00	—	—	—	
	72	0	7.60	8.53	3.62	—	—	—	
3a	0	35	1.85	0	—	1.05	11.9	—	17°
	24	37	1.63	0	—	1.27	12.0	—	
	48	33	2.10	0	—	1.22	11.5	—	
3b	0	35	1.85	0	—	1.05	11.9	—	37°
	24	57	2.92	0	—	5.80	6.65	—	Ein wenig Hämolyse
	48	56	7.34	0	—	6.71	1.80	—	Kräftige Hämolyse
4a	0	34	1.22	0	—	—	12.6	—	17°
	24	36	1.32	0	—	1.42	11.7	—	
	48	38	1.54	0	—	2.71	9.95	—	
	72	40	2.82	0	—	4.57	6.81	—	Ein wenig Hämolyse
4b	0	34	1.22	0	—	—	12.6	—	37°
	24	46	3.78	0	—	8.96	1.50	—	
	48	55	7.47	0	—	6.44	0	—	Ein wenig Hämolyse
5a	0	42	2.56	0	2.98	1.23	13.7	4.11	17°
	24	41	2.18	0	3.32	3.23	12.5	3.01	
	48	44	1.89	0.25	3.62	4.44	11.4	3.19	Ein wenig Hämolyse
5b	0	42	2.56	0	2.98	1.23	13.7	4.11	37°
	24	13	13.5	4.25	4.11	2.50	2.35	1.97	Kräftige Hämolyse
	48	0	22.4	2.49	1.44	—	—	—	Vollständige Hämolyse
6a	0	36	1.86	0	1.70	0.53	12.3	4.51	17°
	24	37	1.92	0	1.55	1.18	10.6	4.22	
	48	37	2.13	0.21	1.01	2.62	9.74	2.20	Ein wenig Hämolyse
6b	0	36	1.86	0	1.70	0.53	12.3	4.51	37°
	24	45	4.95	0	—	7.65	1.00	—	Hämolyse
	48	0	18.6	0	2.60	—	—	—	Vollständige Hämolyse
7	0	30	2.40	0	—	0.59	17.5	—	37°
	24	39	5.00	2.28	—	7.78	5.4	—	Hämolyse
	48	0	17.05	4.25	—	—	—	—	Vollständige Hämolyse

Tagen beginnt allmählich eine Hämolyse der Blutkörperchen stattzufinden, und hierbei tritt der Ester-P ins Plasma über und die Menge an anorganischem P nimmt in demselben zu. Mit der Ausbreitung der Hämolyse auf den grösseren Teil der Blutkörperchen steigt die Hydrolyseschnelligkeit und erreicht in gewissen Fällen relativ hohe Werte. (1 a, 2 a, 4 a).

Bei 37° ist die Hydrolysegeschwindigkeit schon innerhalb 24 Stunden hoch, auch wenn eine offensichtliche Hämolyse noch nicht stattgefunden hat. Die Menge anorganisch gebundenen Phosphors, die während der ersten 24 Stunden frei wurde, variierte zwischen 44 und 92 Prozent der ursprünglichen Quantität. In Versuch 1 b trat keine Zunahme der Phosphorverbindungen im Plasma nach 7 Stunden ein, was beweist, dass sich die Permeabilität erst ändern muss. Nach 48 Stunden waren die Phosphorsäureester in einigen Fällen vollständig hydrolysiert worden (4 b, 6 b). Schon nach 24 Stunden war der Phosphorgehalt im Plasma gestiegen, was darauf beruht, dass die Phosphationen aus den Erythrozyten je nach dem Grade diffundierten, in welchem die Konzentration zunahm.

In den Fällen 3 b, 5 b und 6 b war nach 48 Stunden langer Erwärmung die totale Menge an säurelöslichem P gestiegen, was auf ein Freiwerden von Lipoidphosphor hindeutet.

Versuch 2 c zeigt den Verlauf der Phosphorsäurehydrolyse nach der Hämolyse mittels Saponins. In diesem Falle setzte die Hydrolyse unmittelbar ein, obgleich die Blutprobe bei 17° aufbewahrt wurde. In 24 Stunden waren ungefähr 23 Prozent der Phosphorsäureester gespalten worden, im unhämolysierten Blut dagegen nur 11.5 Prozent, was also einer halb so grossen Hydrolyseschnelligkeit entspricht.

In einigen Fällen wurde auch die Phosphormonoesterasewirkung bestimmt. Es ergab sich, dass die Phosphatasewirkung in den Erythrozyten abnimmt, aber im Plasma im Zusammenhang mit der Hämolyse ansteigt. Die totale Phosphatasemenge ändert sich im Totalblut anfangs nur unbedeutend, sinkt aber bei längerer Aufbewahrung. Dieses zeigt, dass die Phosphatasewirkung, die bei der Analyse zum Ausdruck kommt, nicht der Enzymmenge entspricht, welche im Blut zur Wirkung gelangen kann. Hierbei ist jedoch zu beachten, dass gewisse Phosphorsäureester von dem hier bestimmten Enzym nicht gespalten werden. Das Pyrophosphat wird von der Pyrophosphatase hydrolysiert, aber diese Art von Estern bildet nur ungefähr 10 Prozent der totalen Ester-

menge. Die Phosphormonoesterase im intakten Blut scheint sich deshalb in einem solchen Zustande zu befinden, dass ihre Wirkung nicht zu ihrem vollen Recht kommt, aber mit der Zunahme der Permeabilität der Blutkörpermembran und mit eintretender Hämolyse kann sie ihren vollen Einfluss entwickeln.

Im Versuch mit Saponinhämolyse erhielt ich bei der Bestimmung der Phosphatasewirkung einen etwas höheren Wert als bei der Bestimmung in unverändertem Blut. Der Unterschied 0.9 B. E. ist jedoch nicht so gross, dass er die Verschiedenheit in der Hydrolyseschnelligkeit zu erklären vermag, sondern dürfte einem Versuchsfehler zuzuschreiben sein.

Tabelle 2.

Die Phosphormonoesterase in Bodansky-Einheiten (B. E.).

Nr.	Nach BODANSKY	In physiol. Kochsalzlös.	Bemerkungen
2	5.44	5.14	Blut
8a	5.52	5.52	Blut
8b	6.85	6.71	Blutkörperp.
9a	10.29	—	Blutkörperp.
9b	8.91	—	Hämolysat.
10a	9.68	9.92	Blut
10b	10.86	—	Blutkörperp.
10c	7.18	—	Hämolysat.

Tabelle 2 zeigt das Resultat der Bestimmungen der Phosphormonoesterasewirkung im Blut, teils auf gewöhnliche Weise nach BODANSKY, teils nach derselben Methode, aber mit einem Zusatz von 0.8 Proz. NaCl. Bei der ersteren Versuchsmethodik tritt eine Hämolyse der Blutkörperchen ein, im letzteren Falle nicht. In einigen Fällen wurde die Phosphatasewirkung direkt in einer Aufschwemmung mittels physiologischer Kochsalzlösung gewaschener Erythrozyten bestimmt. In allen diesen Fällen erhielt ich innerhalb der Versuchsfehler dieselben Werte. Die in den Blutkörperchen vorhandene Phosphatase kann also auf die Glycerinphosphorsäure der Reaktionsmischung ihre volle Wirkung entfalten, ohne dass die Blutkörperchen hämolysiert werden. Dieses deutet darauf hin, dass sich die Phosphatase auf der Oberfläche der Blutkörperchen befindet und mit dem umgebenden Medium in näherem Kontakt steht als mit dem Inhalt der Blutkörperchen. Die Versuche 9 b und 10 c zeigten einige Fälle, in welchen die Phosphatasewirkung bestimmt wurde, nachdem die Blutkörper-

chen mit destilliertem Wasser hämolysiert und die Stromata abfiltriert worden waren. In diesen Versuchen fand sich alle Phosphatase im Hämolysat. Bei der Hämolyse wird die Phosphatase also aus der Blutkörpermembran freigemacht.

Da es galt, die Veränderungen, denen das Lecithin anheimfällt, analytisch festzustellen, bestimmte ich die Verminderung im Lipoidphosphorgehalt. Dieses bildet in erster Linie einen Masstab für die Abspaltung der beiden Fettsäuren aus dem Lecithinmolekül, also für die Wirkung der Lecithinase B. Eine andauernde Zerteilung unter Freiwerden von Phosphorsäure gemäss den Formeln auf Seite 126 ist schwieriger nachzuweisen, da man nicht entscheiden kann, ob die freigewordene Phosphorsäure vom Lecithin oder von anderen Phosphorsäureestern her stammt. In gewissen Fällen liess sich jedoch eine Zunahme der Menge sog. säurelöslichen Phosphors nach drei Tage langer Behandlung nachweisen, und diese Steigerung muss auf vorher vorhandenem Lecithinphosphor beruhen. Eine chemische Bestimmung von Lysolecithin lässt sich in geringeren Blutmengen ebenfalls nicht ausführen. Die Bildung von Lysolecithin ist mit der Veränderung der Form der Erythrozyten und dem Eintritt der Hämolyse als festgestellt zu betrachten.

Eine Abspaltung der Fettsäuren kann ebenfalls analytisch durch Titrierung von Alkoholätherextrakt nachgewiesen werden. In einigen Fällen wurden solche Bestimmungen auch ausgeführt. Die freigewordenen Fettsäuren können jedoch nicht nur vom Lecithin her stammen, sondern auch von Neutralfett und Cholesterinestern, weshalb diese Bestimmung keinen Masstab allein für die Zerteilung des Lecithins bilden kann.

Bei 17° liess sich in Pferdeblut innerhalb 3 Tage keine Lecithinspaltung mit Sicherheit nachweisen. Figur 3 zeigt einige Beispiele von Lecithinanalysen in Blut, das bei 38° aufbewahrt wurde. Die Hydrolysegeschwindigkeit variierte in verschiedenen Proben und steht in gewissen Relation zu der Hämolysesgeschwindigkeit. Die Versuche 4 und 13 z. B. zeigten nur wenig Hämolyse, die Versuche 5, 6 und 11 hämolysierten dagegen schnell. Die Spaltung des Lecithins findet sowohl im Plasma wie in den Erythrozyten statt.

Der Gehalt an alkoholätherlöslichen Säuren stieg in Blutproben, die auf Körpertemperatur erwärmt wurden. Die Zunahme fand sowohl im Plasma als auch in den Blutkörperchen statt und nahm mit dem Hämolysedegrade zu (Tab. 3). Der pH-Wert des Plasmas zeigte eine schwache Steigerung.

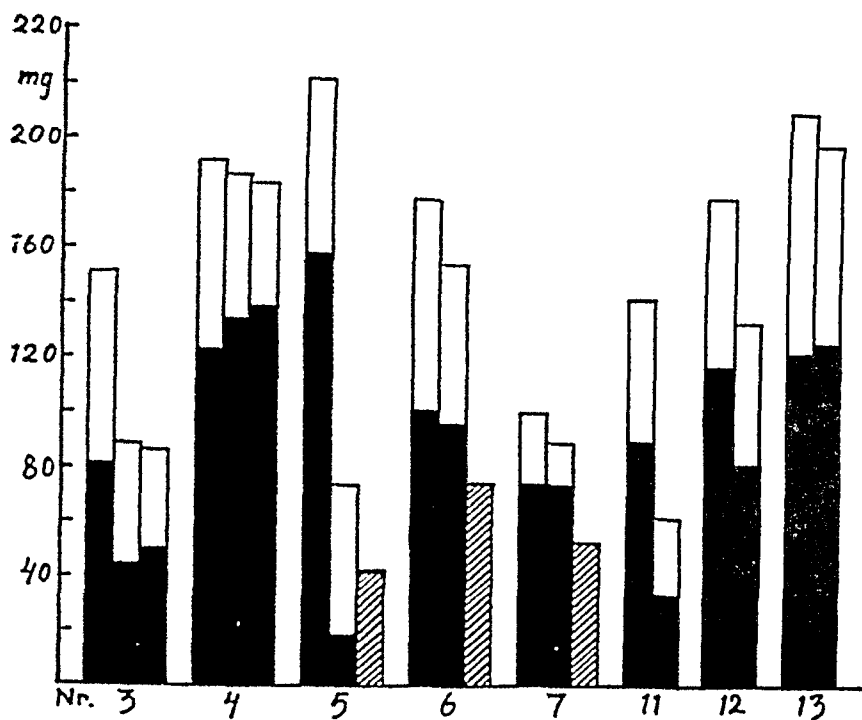


Fig. 3. Veränderungen in der Lecithinmenge. Der gefüllte Teil der Pfeiler gibt die Menge in den Blutkörperchen an, der ungefüllte die Menge im Plasma von 100 ml Blut vor der Erwärmung und nach 24 bzw. 48 Stunden langer Erwärmung auf 37°.

Tabelle 3.

Nr.	Stunden	ml 0.1 n NaOH pr 100 ml		pH im Plasma
		Blut	Plasma	
5b	0	8.5	3.9	7.50
	24	11.7	10.9	8.00
	48	17.0	—	7.35
6b	0	8.1	5.4	7.00
	24	10.2	6.3	7.50
	48	28.8	—	8.00

Zusammenfassung.

Die Arbeit behandelt die Untersuchung der Stabilität organischer Phosphorverbindungen und von Phosphatase im Pferdeblut bei dessen Aufbewahrung in vitro bei 17° und 37° C während eines bis mehrerer Tage.

1. Bei 17° fallen die Phosphorverbindungen im Pferdeblut in 1—2 Tagen nur geringeren Veränderungen anheim. Allmählich tritt Hämolyse ein, und je nach dem Grade ihres Fortschreitens beginnen die organischen Phosphorsäureester in den Erythrozyten unter Abspaltung von anorganischen Phosphaten hydrolysiert zu werden.

2. Bei 37° ändert sich die Form des Blutkörperchens von der münzenähnlichen zur sphärischen Form, wodurch die Hämatokritwerte in 24 Stunden durchschnittlich im Verhältnis 1:1.6 steigen. Die Permeabilität der Blutkörpermembran nimmt zu, so dass K⁺, Na⁺, Ca⁺⁺, Cl⁻ und PO₄^{'''} dieselbe ungehindert passieren können. Bei längerer Aufbewahrung bei 37° tritt Hämolyse ein, die in der Regel nach 3 Tagen vollständig ist.

3. Schon während der ersten 24 Stunden findet eine kräftige Hydrolyse der organischen Phosphorsäureester statt, und nach 3 Tagen ist diese oft vollständig.

4. Auch die Lecithine werden zerteilt, wobei teils die Fettsäuren, teils die Phosphorsäure frei werden.

5. Phosphormonoesterase kommt sowohl in den Erythrozyten als auch im Plasma vor. Das in den Erythrozyten vorhandene Enzym beeinflusst eine Glycerinphosphorsäure enthaltende Reaktionsmischung gleich kräftig, sei es dass die Blutkörperchen bei der Analyse hämolysiert sind oder nicht. Dagegen scheinen die eigenen organischen Phosphorsäureester der Erythrozyten nicht angegriffen zu werden, bevor die Blutkörperchen gewissen Veränderungen anheimgefallen sind, die zu Hämolyse führen.

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Studies on the Muscular Physiology of the Genital Tract.

I. The Spontaneous Tonus of the Uterine Muscle; Its Dependence on Hormonic Factors.¹

By

SUNE GENELL.

Our ideas concerning the importance of the sex hormones for the tonus of the uterine muscle are chiefly based on solitary observations that are not of a nature to admit of statistical treatment. Certainly the baseline of the curve obtained in the Magnus-Kehrer experiment can give some idea of myometrial tonus, but single observations have very limited value, several widely variable factors being involved, such as the load applied, the composition of the serum saline solution and others. Most reports give no particulars of these factors, and hence the observations made are not comparable. There are however solitary observations which tend to show that oestrin exercises a tonus-depressing action on uterine muscle, at any rate in some animals, and it would therefore appear desirable to determine whether this is the case. The present investigation has been carried out on *rats*.

Method: A uterine cornu is extirpated under ether narcosis and, without being cut up, is disposed as a longitudinal preparation by the routine method applicable to surviving organs. Serum saline solution is used of the following composition: NaCl 8, KCl 0.42, CaCl₂ 0.24, MgCl₂ 0.005, NaHCO₃ 1, glucose 0.5 per 1000 c.c. Through this fluid is bubbled oxygen with 5 % CO₂. The kymograph is turned by hand at certain intervals. After the preparation has been mounted, it performs its initial contraction, then relaxes, and, when the recording pen begins to rise again at the second contraction, the kymograph is manually turned about 1 centi-

¹ Received 17 July 1940.

metre. Five minutes later the second turn is made, after which the kymograph is turned every fifth minute for half an hour and thereupon every tenth minute for $2\frac{1}{2}$ hours. The "curve" obtained in this way consists of vertical curved lines, the lower points of which denote the maximum relaxation, the upper points the maximum contraction, during the respective intervals of time. The first curve (denoted "0 min.") represents only the initial contraction and the relaxation following upon it. The initial contraction was the highest of all recorded during the experiments, except in two cases.

A horizontal line is drawn through the top point of the initial contraction curve, and the distances vertically from this line to the relaxation maxima (i. e. the lower points of the curved lines) during the respective intervals of time are measured and tabulated. When the pen has a stroke ratio of 1 : 1, the true values are obtained. The mean of the relaxation values within a certain series of experiments is calculated for each time interval. A graphical record of these values will give a curve that represents the degree of relaxation of the cornu uteri during the three hours the experiment has lasted. Tonus in unstriated muscle is defined by EVANS as "a resistance of its substance to extension". Consequently, the relaxation will be less the higher the tonus is. The relaxation curve described above will therefore give a picture of the state of tonus of the longitudinal muscle of a surviving cornu uteri.

All the factors involved in the experiments were standardized as far as possible, the time selected for the experiments, operative technique, technique and time taken for the preparation, etc. Identically the same instrument, the same recording pen and the same load, balanced so that the pen just had overweight, were employed. The length of time required for the investigation necessitated the use of two kymographs and hence two pens. The very slight difference between the two instruments consisted in the load being slightly larger in one of them. One half of each series of experiments were made with the heavier loaded instrument (group A), one half with the lighter loaded (group B). Hence, although there is a distinct difference between groups A and B (the figures are published in GENELL, 1937), no allowance need be made for this difference on comparing the different means.

Each animal was used twice. The two uterine cornua were taken in different sexual phases, but both were examined in the same apparatus. This gave rise to still another grouping of the material:

Table 1.

Pro				Pos				Met				Neg 4			
Animal	T = 0 m.	T = 60 m.	T = 120 m.	T = 180 m.	Animal	T = 0 m.	T = 60 m.	T = 120 m.	T = 180 m.	Animal	T = 0 m.	T = 60 m.	T = 120 m.	T = 180 m.	
737	22.0	37.0	37.5	38.0	749	17.5	21.5	22.5	22.5	737	7.5	11.0	12.5	11.5	
750	31.5	44.5	46.5	48.0	803	30.0	33.0	33.5	34.5	750	14.0	25.0	27.5	27.0	
736.	16.0	20.0	21.5	22.5	762	19.5	21.0	21.5	21.0	736	16.0	17.5	19.0	19.5	
751	26.5	37.5	40.0	41.5	761	22.5	23.0	22.5	23.5	751	10.0	18.5	20.5	21.0	
723	34.0	38.0	39.5	40.0	784	26.0	29.5	29.5	30.0	723	21.5	23.0	22.0	22.0	
759	20.0	29.0	29.5	30.0	793	18.0	23.5	24.5	26.0	759	17.5	20.5	21.0	20.0	
747	26.0	33.0	33.5	35.5	775	16.5	23.5	24.0	24.5	747	17.5	18.0	14.0	8.5	
760	24.0	27.0	27.0	27.5	786	10.5	16.0	15.0	13.5	760	19.0	20.5	21.0	21.5	
749	24.0	33.5	34.5	35.0	763	30.5	34.5	35.0	36.0	763	16.0	19.0	18.5	17.5	
803	35.0	37.5	39.0	40.5	788	25.0	26.5	27.0	27.0	788	14.5	18.5	17.0	15.5	
762	26.5	31.5	32.0	32.5	774	19.5	27.5	27.5	28.5	774	11.0	15.5	14.0	11.0	
761	19.5	27.0	28.0	28.5	811	22.0	26.0	26.5	27.0	811	8.5	13.5	15.5	16.0	
784	22.0	26.5	27.5	28.0	776	16.0	25.5	26.0	26.0	776	9.5	18.0	17.5	16.0	
793	7.5	11.5	12.5	13.5	800	20.0	28.0	29.5	30.0	800	17.5	23.5	24.5	24.5	
775	27.0	31.0	31.5	32.0	782	20.5	19.5	21.5	21.5	782	7.0	13.0	12.5	9.5	
786	7.5	15.5	16.0	17.0	805	13.5	23.0	24.5	25.5	805	7.0	15.0	14.5	14.0	
753	25.5	30.0	30.5	31.0	757	29.0	32.0	33.0	34.0	773	16.5	22.5	23.5	24.0	
779	23.5	26.0	26.5	27.0	804	24.5	24.5	26.5	27.0	806	13.0	20.5	20.5	20.5	
787	11.0	21.0	24.0	24.0	769	14.5	21.0	23.5	23.0	758	12.0	21.0	22.0	22.0	
783	14.5	23.5	24.5	24.5	772	16.5	22.0	22.5	20.5	756	23.0	24.0	24.5	23.5	
765	23.5	25.5	26.5	26.5	767	22.0	27.0	27.5	28.5	778	13.5	21.5	18.5	13.5	
768	20.0	23.5	24.0	25.0	825	21.5	25.5	25.5	26.0	789	15.5	19.0	19.0	18.0	
764	11.0	13.5	14.5	15.0	755	18.5	23.0	23.5	24.0	781	10.0	17.5	18.0	16.5	
766	9.5	19.5	20.0	20.5	794	10.0	21.5	22.5	22.5	820	8.0	13.0	14.0	12.5	
Means	21.1	27.6	28.6	29.8		20.2	25.0	25.7	25.9		13.6	18.7	18.8	17.7	
whole mat.															
Series I	22.9	30.7	32.0	32.8		21.0	25.8	26.4	26.4		15.1	20.0	19.8	18.8	
Series II	19.3	21.6	23.2	25.9		19.3	24.3	25.0	25.5		12.0	17.5	17.8	16.6	
Square of Stand. dev.	61.6	63.2	71.9	74.9		20.6	18.6	19.4	24.4		21.1	14.2	16.6	25.7	

group I, in which the cornu was taken first, group II, in which it was taken last. The difference between these two groups lies in the possibility of the second cornu being affected by the first laparotomy. In fact, the figures (Table 1) show a distinct difference, those of group II being uniformly lower than those of group I.

Variations in Myometrial Tonus during the Sex Cycle.

The number of animals employed was 48. Fig. 1. gives the curves for the four sexual phases [denoted *pro* (24), *pos* (24), *met* (24), *neg* 4 (24). Each value on the curve represents the mean of 24 determinations.

An extract from the data upon which the curves are based is given in Table 1, in which the relaxation values obtained from each of the 96 experiments are furnished for four different points of time, viz. 0, 60, 120, and 180 minutes.

These values must be regarded as highly interdependent, which implies that the differences between the means of the different series at the different times will be mainly the same.

The square of the dispersion in each series, calculated by the formula

$$\gamma_2 = \frac{1}{n-1} \sum (x-m)^2,$$

is given in Table 1 (Square of the Standard Deviation).

The values of γ vary from series to series, though scarcely more than may be considered as due to pure chance. The mean of all the values is 37, and the dispersion or standard error of the single observation will therefore be 6.1.

The means of the values obtained for the different sexual phases are given in Table 2. The standard deviation of the differences

Table 2.

	T = 0	T = 60	T = 120	T = 180
Pro	21.1	27.6	28.6	29.3
Pos	20.2	25.0	25.7	25.9
Met	13.6	18.7	18.8	17.7
Neg 4	19.9	20.2	21.9	22.1

between different sexual phases is 1.7. Thus, there is no significant difference between pro-oestrus and oestrus or between met-oestrus and dioestrus. The other differences are significant.

Myometrial Tonus in Castrated Animals and in Oestrin-treated Castrates.

The number of animals employed was 24. The castration was always performed in the met-oestrous phase. On the day the first cornu was taken into the experiment, the animal was administered a certain amount of oestrin immediately after the operation. A certain time after that, the second cornu was taken into the experiment. The material includes two series, described as *Old Series* and *New Series*, which are not perfectly alike.

Old Series. Cornu I was extirpated a varying number of days (6th to 12th) after the castration. After the operation the animals were given intramuscularly 5000 I.U. of oestrin in 0.1 cc oil solution. Cornu II was then taken on the day the vaginal smear presented a pro-oestrous picture, about 48 hours after the hormone injection.

New Series. Cornu I was always taken on the 6th day after the castration. After the operation the animals were given subcutaneously 300 I.U. of oestrin in an aqueous solution of alcohol. Cornu II was taken 24 hours later, when the vaginal smear exhibited a commencing pro-oestrous picture and the uterine cornu appeared distinctly vitreous.

The data selected for statistical treatment are submitted in Table 3, in which the relaxation values obtained from each of the 48 experiments are given for four points of time, 0, 60, 120 and 180 minutes, together with the means of these values. The table also contains the means of the values divided between the two somewhat different series, the old series and the new series, as well as the calculated square of the standard deviation. The standard error of the single observation is 4.4. The standard errors of the means for a mean of 24 and 12 individuals respectively will therefore be 0.9 and 1.3 respectively.

Between the old series and the new series there is a difference as regards the means for the untreated castrates in the group. It is however not a significant one and manifestly depends upon chance.

A comparison of the fall in relaxation values from the series of

Table 3.

A n i m a l	Untreated castrates				Treated castrates				
	T = 0	T = 60	T = 120	T = 180	T = 0	T = 60	T = 120	T = 180	
802	13.0	20.5	21.5	21.5	25.5	32.0	32.5	33.0	
812	3.0	11.5	12.0	13.0	12.5	19.0	20.5	21.0	
831	10.0	18.5	18.5	18.0	23.5	28.0	28.5	29.5	
843	16.5	21.0	22.0	20.0	17.5	22.5	23.0	23.5	
845	14.5	21.0	23.0	20.5	24.0	28.0	28.0	28.5	
851	9.0	16.0	16.0	14.0	28.0	29.0	29.0	29.5	
785	7.0	16.0	15.5	15.5	12.5	18.0	18.0	17.5	
807	6.5	16.5	14.5	12.5	11.5	25.0	27.0	27.0	
825	9.5	16.0	17.5	18.0	12.5	21.5	23.5	25.0	
839	13.0	21.5	21.0	19.0	7.0	23.0	23.5	25.0	
840	11.0	19.0	19.5	19.0	19.0	26.0	26.0	26.0	
848	14.0	18.5	19.0	16.5	13.5	19.0	20.0	20.0	
846	17.0	23.5	24.5	24.0	21.0	24.0	24.5	24.5	
855	14.5	23.0	24.0	24.0	18.0	20.5	21.0	20.5	
876	10.0	14.5	16.5	17.5	31.0	34.0	34.5	35.5	
864	14.0	23.5	25.0	25.5	21.0	26.5	27.0	27.5	
861	16.0	19.0	20.0	21.0	22.0	24.0	24.0	24.5	
880	13.0	22.5	23.5	24.5	31.0	32.0	33.0	33.0	
863	8.0	17.0	21.5	22.0	21.5	22.5	22.5	22.5	
856	19.0	26.0	26.5	24.0	27.0	17.0	17.5	15.0	
859	10.0	21.5	21.0	18.5	20.5	25.5	25.5	25.5	
865	20.0	24.0	24.5	25.5	13.5	21.5	21.5	21.5	
869	12.0	16.0	17.0	17.0	20.0	24.0	24.5	26.0	
881	16.5	26.0	27.0	27.5	23.5	24.0	24.5	24.0	
Means {	whole mat.	12.4	19.7	20.5	19.9	19.9	24.4	25.0	25.2
	Old series .	10.6	18.0	18.3	17.3	17.3	24.3	25.0	25.5
	New series .	14.2	21.4	22.6	22.6	22.5	24.6	25.0	25.0
Square of Stand. dev.	17.2	14.2	15.7	17.1	40.4	20.0	19.7	23.6	

untreated castrates to the series of treated castrates gives the following mean values:

Old Series 7.0 ± 1.8

New Series 4.1 ± 1.8

As will be seen, this difference may be due to pure chance. The old and new series can therefore be lumped together without disadvantage.

The values obtained for group II (cornu II) are, on an average, 2.9 below those for group I (cornu I). With regard to the non-castrated animals in different sexual phases (Table 1), the series consist half of I-determinations, half of II-determinations. As regards the castrates, however, the series consist of either only I-determinations (the untreated) or only II-determinations (the treated). For interserial comparison, therefore, the values for the untreated castrates (which all belong to group I) must be reduced by 1.5 (strictly one-half of 2.9), and the values for the treated castrates (which all belong to group II) increased by 1.5.

Table 4.

	T = 0	T = 60	T = 120	T = 180
Untreated	10.9	18.2	19.0	18.4
Treated	21.4	25.9	26.5	26.7

Table 4 contains the values lumped together and corrected in the manner described. The difference between the untreated and treated series is significant. The whole material, corrected as indicated above, is graphically represented in the curves "Untr. Castrates (24)" and "Tr. Castrates (24)" in Fig. 1.

Discussion. E. KEHRER appears to be the first to have pointed out the occurrence of tonus changes in the uterus. He adopts the terminological conceptions derived from special investigations on the phenomenon of tonus, and after him the term "tonus" often occurs in the literature dealing with myometrial motility. Almost equally often no definition or description is given of what the author in question means by the term. In reports on the registration of the motility of the surviving organ, references are made to increased or diminished tonus, usually as a result of drug action in one or the other direction. To distinguish during observation of the organ *in situ* between form-changes of a tonic nature and such caused by active contractions is manifestly impossible. As a consequence there has been some confusion of terms in the literature, alterations in the state of contraction having been

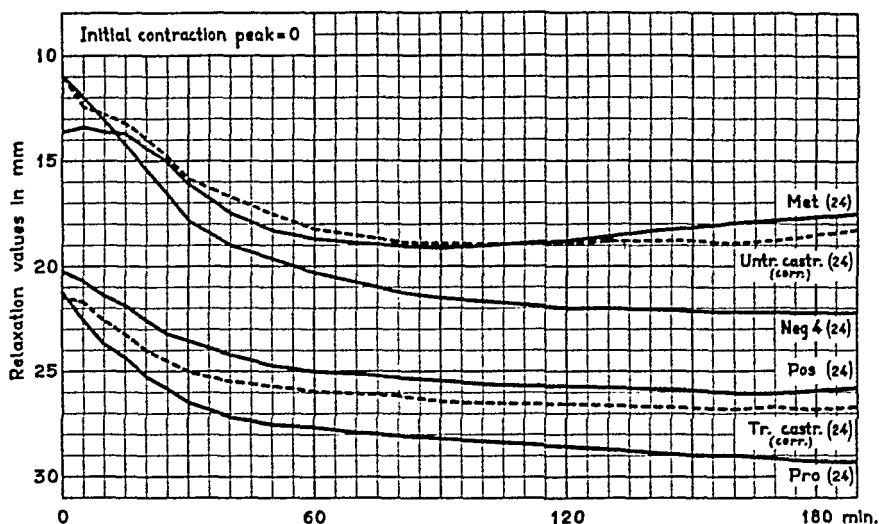


Fig. 1. State of tonus in the longitudinal muscle of the uterms during 180 minutes. The curves have reference to the four sexual phases as well as to untreated and oestrin-treated castrates, 24 animals in each group.

confounded with changes in the state of tonus and the two states having sometimes been regarded as identical.

Starting from a given definition of the term "tonus" (p. 140), the present investigation is an attempt to gather data suitable for statistical treatment and indicative of the spontaneous tonus of the uterine muscle and its dependence on hormonal factors.

The curves in Fig. 1 give the relaxation values for the longitudinal muscle of the surviving cornu uteri between 0 and 180 minutes for the four sexual phases and for untreated and oestrin-treated castrates. The degree of relaxation being dependent on the resistance of the muscle to extension (here = the experimental load), a curve should mirror the state of tonus of the longitudinal muscle during the stated space of time for the respective phase, condition after castration, or condition after oestrin treatment of castrates. If EVANS' previously quoted definition of tonus is accepted, the curves may be denoted as "*Tonus Curves*".

As was made clear in the statistical discussion above, there is no significant difference between the tonus curves for pro-oestrus and oestrus, nor between those for met-oestrus and dioestrus. Since the pro-oestrous and oestrous phases coincide with the maturation and rupture of the follicles, the increment of oestrin must be highest during these stages of the sex cycle. In the met-oestrous and dioestrous phases the corpora lutea ovulationis have

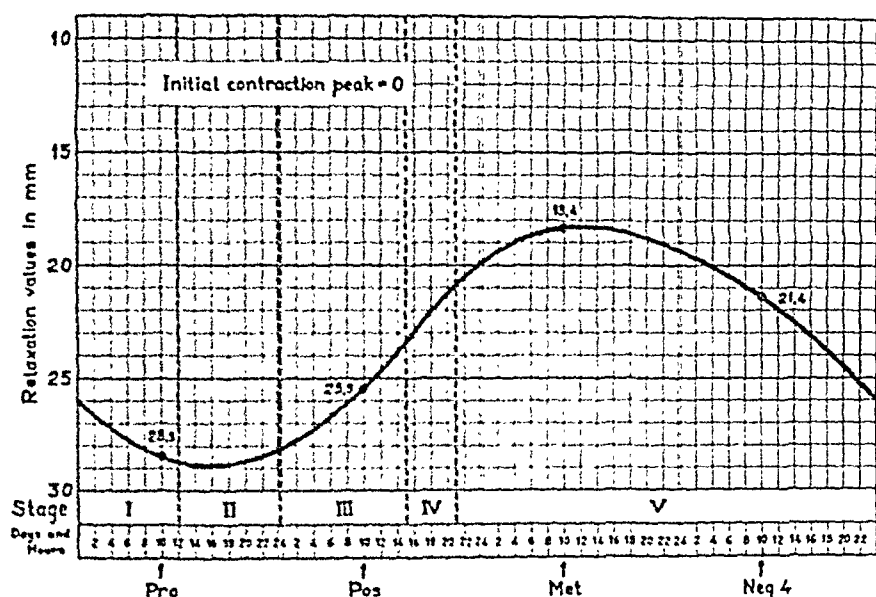


Fig. 2. Variations in the tonus of the longitudinal muscle of the uterus during a sexual cycle. (Stage I—V = sexual phases according to LONG and EVANS, 1922. Pro-Neg 4 = sexual phases according to the author's own division of the sexual cycle. Pro = pro-oestrus, pos = oestrus, met = met-oestrus, neg 4 = dioestrus.) The figures are the means of the values found for 60, 120, and 180 minutes.

been formed. New follicles do not begin to grow until towards the end of dioestrus. The oestrin increment is therefore lowest in these stages. For the purpose of this discussion it is therefore practical to place pro-oestrus and oestrus together under the common head of "*Positive Phase*" and, similarly, met-oestrus and dioestrus under the head of "*Negative Phase*". This grouping is biologically motivated by the oestrin-incretory conditions mentioned above, and also finds support in the fact that the tonus curves for the respective pairs of sexual phases do not exhibit a significant difference, although, of course, this does not rule out the possibility of such a difference being found on the basis of a larger material.

On the other hand, there is a significant difference between one or the other of the tonus curves for the positive phase and one or the other of the tonus curves for the negative phase. The positive phase or *heat phase* is thus characterized by a substantially lower myometrial tonus than the negative phase.

Fig. 2 presents a graphical view of the tonus variations during a sexual cycle. The relaxation values for the respective sexual phases have been computed as the values for 60, 120 and 180 minutes. The value for 0 minute has been omitted for the reason

that at this juncture the organ cannot yet be considered to have adjusted itself to the experimental conditions, a fact that is clearly shown by the curves in Fig. 1.

In Fig. 1 it is observable that in the main the tonus curve for untreated castrates coincides with the tonus curves for the negative phase, and the tonus curve for oestrin-treated castrates in the main with the tonus curves for the positive phase. It is thus possible, by treating castrated animals with oestrin, to produce experimentally the low state of myometrial tonus characteristic of the heat phase.

Summary.

Myometrial tonus varies with the sexual phase. It is low in the heat phase, high in the quiescent phase. The fall of tonus in the heat phase is elicited by the heat hormone of the ovary, oestrin.

Myometrial tonus is not influenced by castration as such. It remains unchanged within that space of time following castration during which muscular atrophy has not yet appeared.

It is probable that the uterine muscle possesses a certain state of normal tonus, maintained largely by unknown factors. We only know that the tonus of the uterus depends on ionic factors, on the osmotic pressure of the blood, and on the hydrogen ion concentration, but little is known of the mechanism through which these factors act. Under the action of oestrin the normal state of tonus falls during the heat phase. The mechanism of this oestrin action is unknown.

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From the Carlsberg Laboratory, Copenhagen.

Method for Rapid Determination of Specific Gravity.¹

By

C. F. JACOBSEN and K. LINDERSTRØM-LANG.

(With 1 fig. in the text.)

The method here described is a simplification of that developed by LINDERSTRØM-LANG and LANZ (1938).

Principle.

A reasonably linear specific gravity gradient is produced in a vertical measuring cylinder (200 cc) by mixing kerosene and bromobenzene in varying proportions. If a drop of a given solution is introduced under the surface, it will fall with diminishing velocity and finally come to rest at a position in the cylinder where the specific gravity of the kerosene-bromobenzene mixture is equal to that of the drop in question.

Before introducing the drop of the solution under investigation, other drops (standard drops) are introduced of potassium chloride solutions of accurately known specific gravity. Naturally these drops will also come to rest at levels where their specific gravities are equal to that of the surrounding medium. Plotting the positions of the potassium chloride drops as ordinates against the corresponding specific gravities as abscissae, a reasonably straight line is obtained in the coordinate system, from which it is possible, knowing the position of the drop of the unknown solution, to read the specific gravity of this solution with considerable accuracy. The specific gravity may also be calculated by linear interpolation.

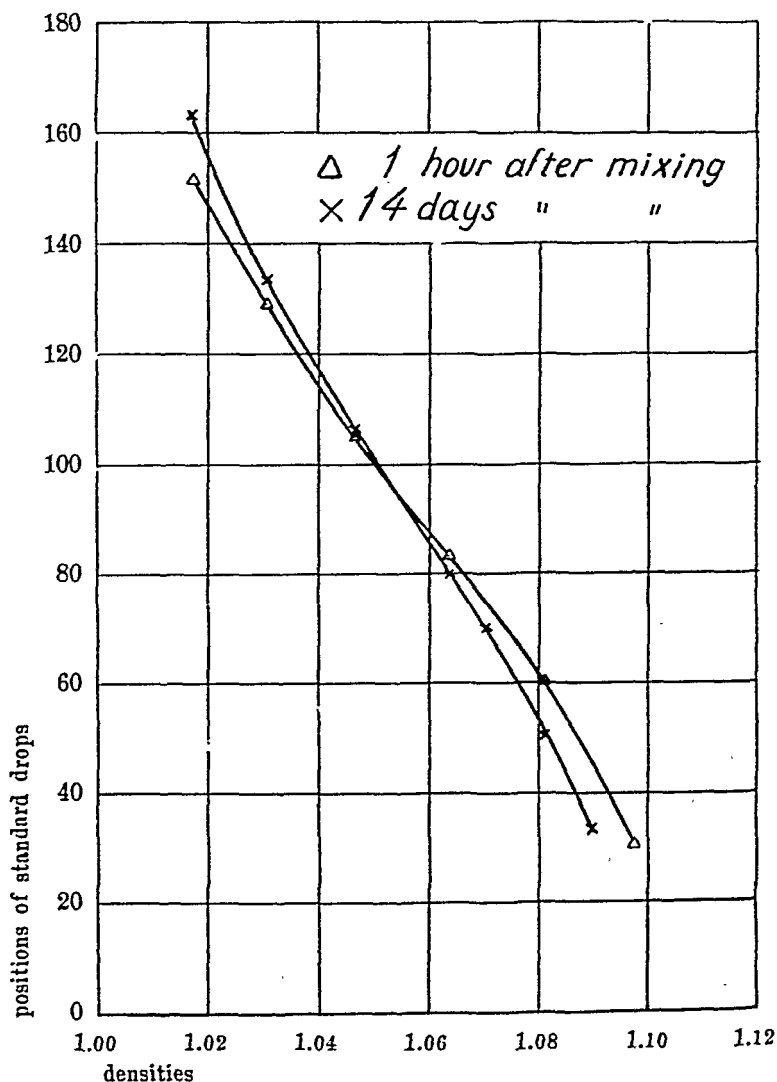
This simple principle is known from the above quoted paper by LINDERSTRØM-LANG and LANZ.

¹ Received 14 July 1940.

The additional facts which justify a separate publication are that for rough density determinations it is unnecessary to place the gradient tube in a thermostat and that the reading of the positions of the drops may be done with the naked eye making use of the scale divisions on the measuring cylinder which serves as gradient tube.

Preparing the Gradient.

If it is the object to determine specific gravities of, say, from 1.03 to 1.11, two kerosene-bromobenzene mixtures are prepared, of the specific gravities abt. 1.01 and 1.12 respectively. 100 cc



of the heaviest mixture is filled into the measuring cylinder, whereupon 100 cc of the lighter mixture is cautiously filled in on top of the heavy one. In order to adjust the gradient, a long spatula is moved up and down through the cylinder and rotated at the same time, until streaks appear at the top and at the bottom. After standing for a short time (30 min.) the gradient tube is ready for use.

Upon standing, the gradient curve becomes flatter because the differences in specific gravity decrease due to currents and diffusion. The end result will be that the specific gravity is the same at all heights. This, however, requires a rather long time. The figure shows the change of the gradient in the course of 14 days for a gradient tube which stood unprotected on a table, exposed to vibrations and drafts.

Making the Determinations.

The standard drops, having specific gravities varying from 1.03 to 1.11 with intervals of 0.02 are introduced by means of a 1 mm³ pipette with rubber tube. (Compare LINDERSTRÖM-LANG and LANZ). In this rough method the drop size is of little importance and may vary 100 per cent without affecting the equilibrium position. After the pipette has been filled with the standard solution and emptied again by blowing, (not of course, into the bottle of the standard solution) a number of three times, it is filled to the mark and dried on the outside with filter paper, without the paper touching the point. The drop is then introduced into the gradient tube by the following method: The point of the pipette is brought to abt. 3 mm below the surface; the drop is forced out by blowing gently through the rubber tube, but does not slip from the point until the pipette is cautiously withdrawn through the surface, while at the same time care is taken to have a low excess pressure in the pipette. It is then possible to introduce a second standard drop, of the same specific gravity, without making the above mentioned three cleaning operations. The next standard is then introduced, after three cleanings. It is obvious that the heaviest standard drops should be introduced first in order to make collisions between the drops less likely.

Drops from the solution to be examined are then introduced in the same manner.

Reading of the height of a drop is made by keeping the eye at the level of the drop, in order to eliminate errors due to parallax.

A number of drops may, of course, be introduced into the gradient tube at the same time, but it is not advisable to use the tube for more than abt. two hours at one time.

All drops are removed when there is no more room for drops to be tested or when abt. two hours have lapsed since the standard drops were introduced. For this purpose a long, thin glass rod, with its point wrapped in moist filter paper, is introduced into the gradient tube; the drops are absorbed by the filter paper as soon as it touches them. The gradient tube is again ready for use after standing for abt. 20 minutes.

The method may, for example, be used in determining the specific gravity of blood. Its accuracy is 0.1 per cent.

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LINDERSTRØM-LANG, K., and H. LANZ, C. R. Lab. Carlsberg, sér. chim. 1938, 21. No 24.

On the Influence of Atropine on some Nicotine-like Actions of Acetylcholine.¹

By

N.-O. ABDON.

(With 4 figures in the text.)

In his fundamental study on the pharmacology of acetylcholine (ac. ch.) DALE (1914) divides its effects in muscarinelike and nicotineline actions. The susceptibility to atropine was considered a main difference between the two kinds of action. The muscarine-like actions were abolished by atropine, while the nicotineline ones were supposed to be uninfluenced. Since then, the nomenclature and classification of DALE have been widely accepted, but with regard to the atropine antagonism the classical definition has been modified *de facto*. Thus, RIESSER and NEUSCHLOSS (1921) showed that atropine abolished the effects of ac. ch. on skeletal muscles of frog, and in 1930 DALE and GADDUM found that atropine prevented or diminished the action of ac. ch. on stripes of cat's denervated diaphragm, suspended in Ringer's solution. FRANK NOTHMANN and HIRSCH-KAUFFMANN (1922) found that scopolamine antagonized contractures of skeletal muscles provoked by intraarterial injections of ac. ch. This result could not be confirmed by DALE and GADDUM (1930).

As a general rule, both kinds of ac. ch. actions are antagonized by atropine, although several exceptions are described. Thus, the action of ac. ch. on the heart of *helix pomatia* (JULLIEN and MORIN, 1931) and on the dorsal muscle of leech (MINZ, 1932) are not influenced. Although atropine antagonizes the ac. ch. effects on striped muscles of frog and rabbit when suspended, it has hitherto not been possible to demonstrate the antagonism on the "quick contractions" of voluntary muscles, provoked by intra-

¹ Received for publication 4 Aug. 1940.

arterial injections of ac. ch., neither in mammals (BROWN, DALE, and FELDBERG, 1936) nor in frog (RAVENTOS, 1937). In the present paper some experiments are reported in which it has been possible to abolish the "quick contractions" with reasonable amounts of atropine. According to BACQ and BROWN (1937) atropine does not antagonize the action of ac. ch. on ganglia.

Although the nicotineline as well as the muscarineline actions are antagonized, there is generally a quantitative difference between the two kinds of actions with regard to the amounts of atropine necessary. Most muscarineline actions are abolished by small amounts of atropine, while most nicotineline ones require so large amounts that the antagonism has to be studied on isolated organs. Many authors are of opinion that this quantitative difference is so great that the action of atropine has still to be accepted as a criterion of a fundamental distinction between muscarineline and nicotineline actions. If one accepts this quantitative difference as proving such a distinction, it would, however, be necessary to disregard the striking regularity in the influence of other agentia on the actions of ac. ch.

Whether an action of ac. ch. is nicotineline or muscarineline, whether it is regarded as inhibiting or stimulating, it is potentiated by drugs as eserine, fluoride, ergotamine, oxalate, citrate, quinine, digitalis glucosides, etc. It is potentiated by degeneration of efferent nerves (degeneration of motor nerve of striped muscle, DALE and GADDUM (1930) *et al.*; of motor nerve of smooth muscle, ROSENBLUETH (1932); and of preganglionic nerve of a ganglion, CANNON and ROSENBLUETH (1936). Also hydrogen ions potentiate the effects of ac. ch. (ANDRUS, 1924, *et al.*). Curarine, on the other hand, antagonizes the action of ac. ch. on striped muscles as well as on the frog's heart (RAVENTOS, 1937).

As to eserine and prostigmine, this regularity is explained by the inhibition of the choline esterase, but the effect of the other agentia cannot at all, or only to a small extent, be explained as an influence on the esterase. Fluoride, ergotamine, and quinine cause in rather large concentrations a certain inhibition of the esterase, but this action seems to be of little importance to their potentiating effect. KAHLSON and UVNÄS (1938) showed that these drugs develop their potentiating effect in concentrations which are too small to exert any inhibiting action on the esterase. They found further that these drugs sensitize the organs to the stable choline compound carbaminoylcholine. In this institute we have

further examined oxalate, citrate, and strophanthin. We found that a concentration of 1:1,000 of these drugs does not inhibit the esterase to any measurable degree, while much weaker solutions sensitize the eserized m. rectus abdominis and the frog's heart to ac. ch. (not published). Nor can the sensitizing effect of hydrogen ions be explained as an influence on the esterase. AHLGREN (1929) found that preparations of the rabbit's gut is markedly sensitized to ac. ch. by moving the pH of the suspension fluid from 7.7 to 7.1, while the results of WAHLQIST (1935) show that variations in the hydrogen ion concentration in the range between pH 8 and pH 7 have no effect on the esterase activity. Nor is the effect of degeneration of efferent nerves caused by decrease of the esterase activity. According to D. NACHMANSOHN the esterase activity of denervated skeletal muscles is increased (not published). It seems, therefore, to be necessary to assume that the majority of the potentiating agents exert their actions by interfering with the mechanism of action of ac. ch.

The regularity in the influence of many agents on the different actions of ac. ch. suggests an essential similarity between the mechanisms of action of muscarinelike and nicotinelike effects. Against this similarity stands the quantitative difference between the various actions of ac. ch. in their relation to atropine. It seems, therefore, to be of a certain interest to study if this quantitative difference in reality signifies a qualitative distinction between muscarinelike and nicotinelike effects. One must, namely, be impressed by the fact that the producing of nicotine-like effects generally requires much larger concentrations of ac. ch. than the producing of muscarinelike effects, and it seems to be possible that those properties of an organ which make a large amount of ac. ch. necessary also make a large amount of atropine necessary for abolishing the ac. ch. effect. CLARK, GADDUM, *et al.* (*vide* CLARK, 1937) have produced mathematical expressions of the quantitative relations of ac. ch.-action and of the antagonism between ac. ch. and atropine. On the basis of those expressions the writer has studied the ratio between ac. ch. and atropine in a series of experiments on various organs of frog.

As described by CLARK (1937), the relation between concentration and effect of ac. ch. follows HITCHCOCK-LANGMUIR's law in all organs studied:

$$K [\text{ac. ch.}] = \frac{y}{100 - y};$$

K is a constant, y is the effect provoked by [ac. ch.] expressed as percentages of the maximal effect which can be provoked by very large amounts of ac. ch. The action of many inhibitors of enzymes has earlier been shown to follow this law, and this fact has been the base of certain theories on the mechanism of action of enzyme poisons. With regard to ac. ch., CLARK makes the same conclusions. He postulates that the point of attack of ac. ch. is located to certain receptors, which react with the ac. ch. molecules according to the law of mass action. The effect of a certain amount of ac. ch. is proportional to the number of receptors, fixing ac. ch. molecules, in relation to the total amount of receptors in the organ. Thus, y , which in CLARK's expression represents the effect of [ac. ch.], also represents the relative number of receptors which have fixed ac. ch. molecules, and 100 represents the maximal effect of ac. ch. as well as the total amount of receptors in the organ.

If one postulates that the ac. ch. receptors of various organs are identical, it would be possible to explain those differences in sensitiveness to ac. ch. of various organs which remain after the influence of cholinesterase has been eliminated in such a way that a less sensitive organ contains a larger number of receptors than a sensitive organ. According to this view, the various amounts of ac. ch. which are necessary for producing the same effect on various organs, *e. g.* 50 p. c. of the maximal effect, should be directly proportional to the absolute number of receptors.

The antagonism between ac. ch. and atropine has been subject to quantitative studies by CLARK (1926, 1927) and GADDUM (1937). Both of them found that the relation between concentration and effect of ac. ch. in atropinized organs also followed the same simple law of HITCHCOCK-LANGMUIR (with the difference only that the value of the constant K is lower after addition of atropine). On the basis of this fact GADDUM (1937) formulated a mathematical expression for the relation between concentration and effect of ac. ch. in the presence of arbitrary amounts of atropine. CLARK (1937) found that this expression was well satisfied by values experimentally found. Both authors made the following interpretation: atropine and ac. ch. compete for the same receptors; the decrease in sensitiveness to ac. ch. is caused by the fixation of atropine at the ac. ch.-receptors, thus blocking the receptors to ac. ch. At the presence of both atropine and ac. ch. a balance is brought about between ac. ch.-molecules, atropine-molecules, free receptors and receptors fixing atropine and ac. ch. respectively.

This means that if a certain effect of ac. ch., e. g. 50 p. c. of the maximal effect, is to be diminished to another fixed effect, e. g. 25 p. c. of the maximal effect, the same number of receptors in relation to the total number of receptors must be fixing atropine, whatever organ is chosen. If one postulates that the receptors of various organs are identical, the amount of atropine which is necessary to diminish the effect of ac. ch. from 50 to 25 p. c. of the maximal effect must be directly proportional to the absolute number of receptors. As the amount of ac. ch. necessary for producing the 50 p. c. effect is also directly proportional to the absolute number of receptors, the amounts of atropine and ac. ch. in question must be directly proportional to each other, i. e. the ratio between the amount of ac. ch. provoking the 50 p. c. effect and the amount of atropine that lowers this effect to 25 p. c. should be constant, whatever organ is chosen.

To test the validity of this discussion I have made determinations of these amounts of ac. ch. and atropine on a series of organs of frog. Muscarinelike as well as nicotinelike effects were examined. The ratio between these amounts of ac. ch. and atropine was found to be fairly constant.

Because of these results I made some experiments on the influence of atropine on "quick contractions" of skeletal muscles provoked by intraarterial injections of ac. ch. and on the transmission between motor nerve and skeletal muscle.

A. The quantitative relation between ac. ch. and atropine in various organs.

Method.

The experiments were made on heart, stomach, intestine, and a series of skeletal muscles of frog (*Rana temporaria*). In all organs the influence of the esterase was abolished by adding eserine sulphate 1:200 000 to the Ringer solution.

The hearts were suspended according to STRAUB and fed with oxygenated Ringer solution. As it was noted that the hearts became more sensitive to ac. ch. when they grew weaker, the hearts were used only as long as the beats had their original heights as registered by an isotonic lever. To avoid anoxia the Straub cannulas were provided with specially short and wide necks. As known, the proportion between the inotropic and the chronotropic effect of ac. ch. and some other inhibiting drugs varies greatly. Even during the very influence of ac. ch. a sudden change in this proportion is often noted. When the inotropic or the chrono-

tropic effect is separately used as a criterion on the magnitude of ac. ch. action — as in these experiments — these conditions may cause a rather great error. Therefore, only those experiments were considered where the hearts reacted solely inotropically or chronotropically.

The other organs were suspended in oxygenated Ringer solution in the ordinary way. The shortenings of the muscles were registered with isotonic levers. In every experiment the height of the maximal ac. ch.-effect was first determined by adding an amount of ac. ch. which was 1,000 times larger than the threshold concentration. The maximal shortening was determined again at the end of every experiment and if the values were not identical the experiment was ignored. The amount of ac. ch. which provoked 50 p. c. of the maximal effect was carefully determined. The amount of atropine sulphate was titrated which was necessary to diminish the effect of ac. ch. from 50 to 25 p. c. of the maximal effect. As mentioned below, atropine takes such a considerable time to develop its full antagonistic action on skeletal muscles that it was not considered proper to test more than two concentrations of atropine on each preparation of skeletal muscles. If the second test was not successful the experiment was not carried on any further.

At the determination of the magnitude of ac. ch.-action it is usual to measure the height of muscle shortening 3 to 5 minutes after the addition of ac. ch. In the present experiments the added amount of ac. ch. was allowed to act until the curve of shortening became asymptotic. In the case of skeletal muscle this ensued after 5—10 minutes and in the case of stomach or intestine preparations after 3—5 minutes. The velocity of development of the contracture of the individual preparation was as a general rule constant, but sometimes it was observed that the velocity changed greatly during the course of experiment. In spite of this change, the same amount of ac. ch. gave the same definitive height as before.

In these experiments I had to determine the effect of a certain amount of atropine after it had developed its antagonistic action completely. RIESSER and NEUSCHLOSS (1922) noted that atropine developed its action on skeletal muscles more slowly than ac. ch., but the literature does not seem to offer any definite statements as to the time required for the complete development of the antagonistic effect. Most observers have arbitrarily treated the preparations with atropine for 5 or 10 minutes before the addition of ac. ch. On preparations of stomach or intestine of frog this time seems to be sufficient, while skeletal muscles have to be exposed to atropine for a considerably longer period before full effect of a certain dose of atropine is achieved. Even in case of a thin muscle as *m. rectus abdominis* of frog the necessary period of time was found to be about 50 minutes (see fig. 1). This was determined by titrating the amount of ac. ch. necessary for

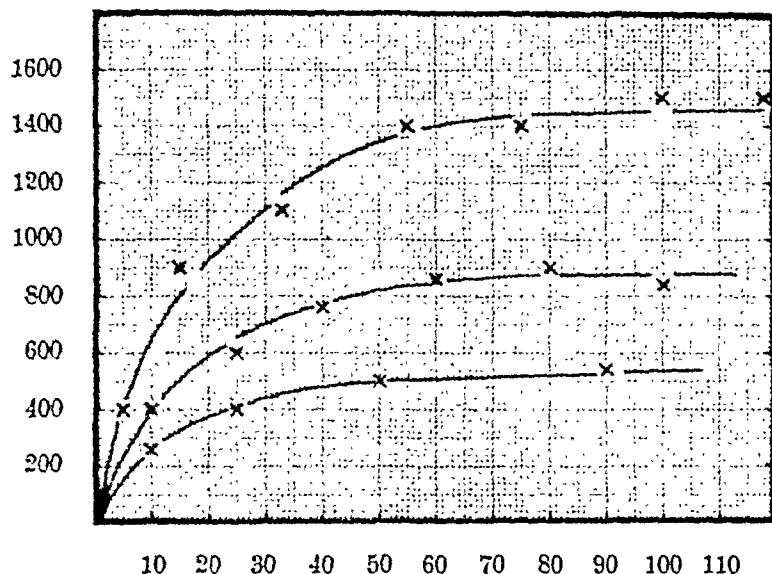


Fig. 1. The development of the ac. ch.-antagonistic action of atropine on m. rectus of frog as a function of time.

x = time in minutes after the addition of atropine

y = the increase of the amount of ac. ch. necessary for provoking 50 p. c. of the maximal effect. (Calculated as percentages.)

The upper curve represents the action of atropine sulphate 1 : 60,000, the middle curve represents the action of atropine sulphate 1 : 120,000, and the lower curve the action of atropine sulphate 1 : 250,000.

provoking a 50 p. c. effect before and at varying intervals after the addition of atropine. In consequence of this finding the skeletal muscles used in the following experiments were exposed to atropine during at least 60 minutes.

Results.

As shown by fig. 2, the amounts of ac. ch. required for giving 50 p. c. of the maximal effect on the different organs vary within nearly 3 powers of 10; from 1 : 200 millions in the case of frog's heart to 1 : 250,000 in the case of m. dorsalis scapulae. As to the hearts, the sensitiveness found in these experiments is in accordance with values found by CLARK (1926) and BEZNAK (1934); as to the skeletal muscles the values agree with those given by WACHHOLDER and LEDEBUR (1930). The amounts of atropine required for diminishing the effect of ac. ch. from 50 p. c. to 25 p. c. of the maximal effect varied on the whole in relation to the

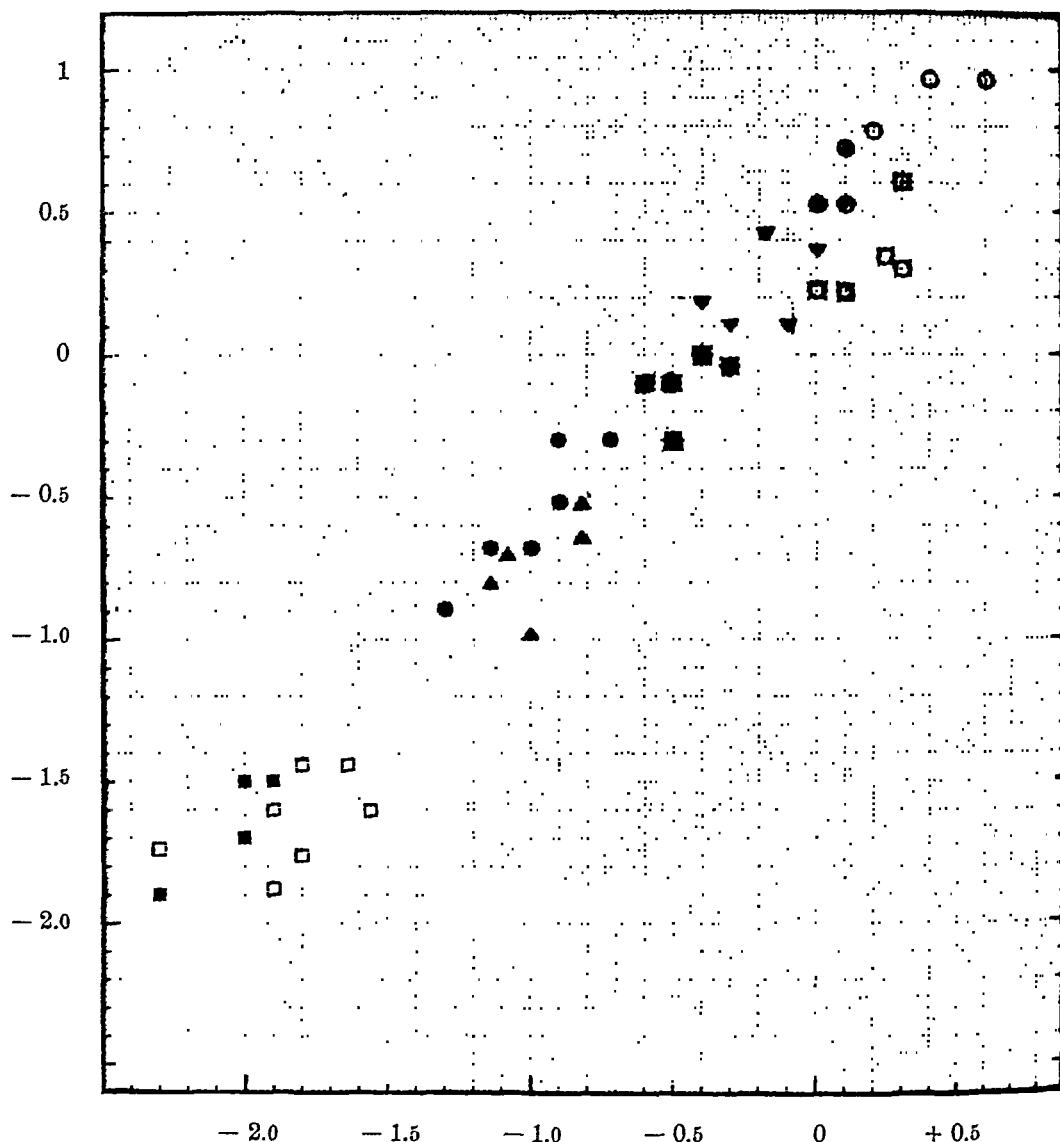


Fig. 2 shows the relation between the amount of ac. ch. Cl necessary for provoking 50 p. c. of the maximal effect in various organs and the amounts of atropine sulphate which diminish the 50 p. c. effect to 25 p. c. of the maximal effect.

x = the logarithm of the concentration of ac. ch. Cl calculated as γ per ml of suspension fluid.

y = the logarithm of the concentration of atropine sulphate as γ per ml of the suspension fluid.

■ = chronotropic effect on heart

□ = inotropic effect on heart

▲ = longitudinal muscles of small intestine

▼ = longitudinal muscles of stomach

● = m. rectus abdominis

● = m. pectoralis abdominis

⊗ = m. sartorius

⊗ = m. gastrocnemius

⊗ = m. dorsalis scapulae

necessary amount of atropine. Although the values of the ration between the amounts of atropine and ac. ch. vary within rather wide limits, the correlation between the amounts in question is beyond doubt, the correlation coefficient being $+0.91 \pm 0.026$. Thus the expression presented in the introduction is satisfied:

$$\frac{(\text{ac. ch.})}{(\text{atropine})} = K;$$

Thus, it may be said that those properties of an organ which make large amounts of ac. ch. necessary for provoking an effect, obviously also make large amounts of atropine necessary for the demonstration of the antagonism, whether the antagonism is studied on muscarinelike or nicotinelike effects. The quantitative differences between nicotinelike and muscarinelike actions with regard to the atropine antagonism do not constitute any real distinction between the actions of ac. ch. The behaviour towards atropine rather augments those similarities between the two actions of ac. ch. which are signified by the uniformity in the influence of many potentiating and antagonistic agents.

B. The antagonistic action of atropine on the "quick ac. ch.-contraction".

As mentioned in the introduction of this paper, it has been shown that the action of ac. ch. on skeletal muscles is abolished by atropine if the muscles are suspended and ac. ch. added to the suspension fluid. The effect of intraarterially injected ac. ch. on skeletal muscles of frog or rabbit is, however, not found to be antagonized. DALE and his co-workers have offered an explanation of this phenomenon that agrees with the theory of humoral transmission. They suppose that atropine forms a barrier surrounding the receptors, which blocks the way to the ac. ch.-molecules which diffuse into the muscle from the suspension fluid. When injected intraarterially, however, the ac. ch. comes into such an intimate relation to the receptors that the atropine barrier need not be passed. The ac. ch. that is liberated at the stimulation of motor nerves of striped muscles also comes into that intimate relation to the receptors and is therefore not antagonized by atropine, while ac. ch. liberated by the heart vagus acts after diffusion into the surrounding tissues and is antagonized.

It should be noted, however, that this view on the mode of action of atropine does not concord with the quantitative studies of the antagonism between ac. ch. and atropine, which make it probable that atropine acts on the very ac. ch.-receptors.

In the experiments on suspended muscles rather large amounts of atropine have been used. In the case of suspended mammalian muscles DALE and GADDUM (1930) found the effect of ac. ch. to be abolished by atropine sulphate 1 : 3,000 while atropine sulphate 1 : 150,000 only diminished the effect of ac. ch. In the *in vivo* experiments on "quick contractions" atropine was injected intravenously in such amounts that the actual concentration of atropine in the muscles must have been much smaller than in the suspended muscles. In most "quick contraction" experiments, amounts of atropine were used, which were just sufficient to abolish the effect of ac. ch. on heart and blood pressure; in a few experiments 10—20 times larger amounts were used. From the data in fig. 2 it is evident that still larger amounts should have been used. At the calculation of the concentration of atropine in the muscles from the intravenously injected amount it is also necessary to remember that only a slight part of the injected atropine is fixed in the muscles; the main part is fixed by the liver and the kidneys (OELKERS, HAETZ and RINTELN, 1932). It therefore seems possible that the amazing difference between ac. ch. administered to the suspension fluid and ac. ch. administered intravenously with regard to the susceptibility to atropine is due only to the fact that greatly different concentrations of atropine were used in both cases.

Therefore, I have made some experiments on the influence of larger amounts of atropine on the effect of intraarterially injected ac. ch. The experiments were made on m. gastrocnemius of frog and rabbit. In the experiments on frog the gastrocnemius was suspended in Ringer solution and the atropine added to the suspension fluid. In the experiments on rabbits the atropine was injected intravenously. Independent of the way of administration of atropine the "quick contractions" were abolished. The preparations for intraarterial injections were made according to BROWN (1937) and BROWN, DALE and FELDBERG (1936); as to the details of the technique these communications are referred to.

Experiment 3. The influence of atropine on the "quick contractions" of frog's gastrocnemius.

On a large Hungarian rana esculenta the sciatic artery was prepared for injection according to the «close arterial method». The proximal end of m. gastrocnemius was fixed by a pin, stuck through the femur into a small wooden plate, which was then placed in a Petri dish filled with oxygenated Ringer solution. Tendo achilleus was connected to an isometric lever in such a way that m. gastrocnemius was kept immersed in the Ringer solution while the other parts of the frog were not immersed. In these experiments the muscle was not perfused, the oxygen being supplied only through the suspension fluid.

After a series of contractions, provoked by stimulating the sciatic nerve with condenser discharges, 0.2 γ of ac. ch. Cl dissolved in 0.2 ml of Ringer solution was injected into the artery. A "quick contraction" was provoked (see fig. 3 A). Atropine sulphate was

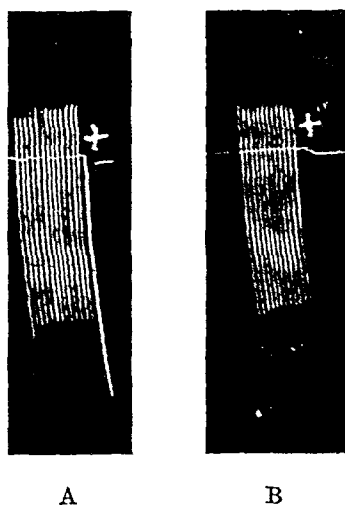


Fig. 3. The influence of atropine on the "quick contraction" of frog's gastrocnemius. At + intraarterial injection of 0.2 γ of ac. ch. Cl. A = before the addition of atropine, B = 45 minutes after the muscle had been immersed in atropine sulphate 1 : 10,000.

added to the suspension fluid to a final concentration of 1 : 10,000. After being exposed to the atropine for 45 minutes the muscle could still be stimulated through the nerve, but an injection of 0.2 γ of ac. ch. Cl into the artery had no effect (see fig. 3 B). Even 25 γ of ac. ch. Cl dissolved in 0.2 ml of Ringer solution gave no contraction of m. gastrocnemius, although this amount was large enough to cause a distant effect, *i. e.* a contracture of mm. recti abdomini and of muscles of the foreleg, which had not been exposed to atropine.

Experiment 4. The influence of atropine on the "quick contractions" of the rabbit's gastrocnemius.

In these experiments the atropine was given by intravenous injection. As it was a priori probable that so large amounts of atropine would be necessary that they would interfere with the brain, the experiments were made on decapitated animals. 4 experiments were made, which gave the same results; one of the experiments is related below.

A rabbit, weighing 1.8 kilos, was anaesthetized with ether. A cannula was inserted in the trachea and artificial respiration was given. Decapitation was made by means of the apparatus used in HEYMANS' laboratory (1932), then no more ether was given. M. gastrocnemius was prepared for intraarterial injection of ac. ch. with maintenance of the natural circulation according to BROWN, DALE, and FELDBERG (1936). The contractions were recorded by means of an isometric lever. For injection of atropine a cannula was inserted into the femoral vein of the other leg.

After a series of contractions, provoked by stimulation of the sciatic nerve with condenser discharges, 10 γ of ac. ch. Cl were injected into the artery, which provoked a "quick contraction"

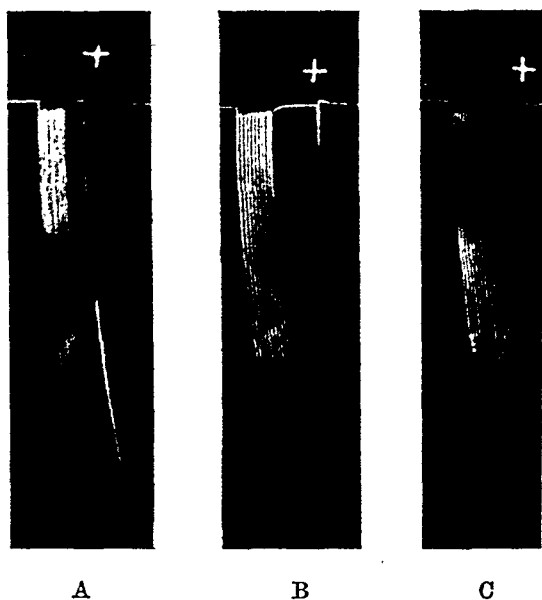


Fig. 4. The influence of atropine on the "quick contraction" of rabbit's gastrocnemius. At + intraarterial injection of 10 γ of ac. ch. Cl. A = before administration of atropine. B = 15 minutes after an intravenous injection of 10 mg of atropine sulphate per kilo of bodyweight, C = 15 minutes after injection of another 10 mg of atropine sulphate per kilo of bodyweight.

of great tension (see fig. 4 A). 18 mg of atropine sulphate (10 mg per kilo bodyweight) were given intravenously and after 15 minutes the injection of ac. ch. was repeated. It still provoked a "contraction", the tension was, however, considerably smaller (see fig. 4 B). Another 18 mg of atropine sulphate were injected and after 15 minutes 10 γ of ac. ch. Cl were injected into the artery, this time without any effect (see fig. 4 C). Then further amounts of atropine were injected, each time 18 mg with an interval of 15 minutes between every injection. After injection of a total amount of 72 mg of atropine sulphate the heart rate was markedly slower and the arterial blood pressure was diminished. After the injection of a total amount of 108 mg the heart ceased to beat, but even now electrical stimulation of the sciatic nerve provoked contractions.

C. The so called "curare-action" of atropine.

In the experiments on the influence of atropine on the "quick ac. ch.-contractions" it was always observed that while abolishing the effect of ac. ch., atropine had no influence on the excitability of the muscle through its nerve. The effect of nerve stimulation was not even influenced by amounts of atropine 4 or 5 times larger than those which abolished the ac. ch.-effect.

To judge from these experiments there seems to exist a certain difference between the effect of ac. ch. and the effect of motor nerve stimulation with regard to their susceptibility to atropine. Previous experiments have, however, shown that large amounts of atropine block the transmission between motor nerve and skeletal muscle (BODKIN, 1862, *et al.*), and some authors count atropine among substances with curare-action (*vide* CUSHNY, 1924). As shown by the experiment below — which is in accordance with data given by CUSHNY (1903) and HAFFNER (1918) — it is necessary to use rather massive concentrations of atropine to demonstrate the "curare-action", and as emphasized below, this "curare-action" is not of the same nature as the ac. ch.-antagonizing effect to atropine.

Experiment 5.

8 nerve-muscle preparations of m. gastrocnemius of frog were made in the usual manner. Two preparations were placed in oxygenated Ringer solution, two in atropine sulphate 1:250, two in atropine

sulphate 1 : 125, and two in atropine sulphate 1 : 75. Every 30 minutes the indirect and direct excitability of the muscles were tested by means of condenser discharges. The result is seen from the scheme below (+ = contraction, — = no contraction).

	hours	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7
control	directly	+	+	+	+	+	+	+	+	+	+	+
	indirectly	+	+	+	+	+	+	+	+	+	+	+
atropine 1 : 250	directly	+	+	+	+	+	+	+	+	+	+	+
	indirectly	+	+	+	+	+	+	+	+	+	+	—
atropine 1 : 125	directly	+	+	+	+	+	+	+	+	+	+	—
	indirectly	+	+	+	+	+	—	—	—	—	—	—
atropine 1 : 75	directly	+	+	+	—	—	—	—	—	—	—	—
	indirectly	+	—	—	—	—	—	—	—	—	—	—

Like similar experiments by other authors, this experiment shows that skeletal muscles, suspended in rather concentrated solutions of atropine, lose their indirect excitability after an interval, the length of which is depending on the concentration of atropine. If atropine is allowed to act on the muscles some further time, the muscles do not answer even to direct stimulation. HAFNER (1918) also studied the curare-action of atropine, added to perfusion fluid. He found that atropine administered this way exerted the "curare-effect" in smaller concentrations, 1 : 1,000—1 : 2,000. Also the direct excitability was abolished after prolonged perfusion.

At those concentrations, which are necessary for provoking the "curare-action" of atropine, many drugs will most probably block the transmission between nerve and skeletal muscle. *A priori* it seems to be likely that this effect of atropine is unspecific. With regard to the theory of humoral transmission it is, however, interesting to state that the "curare-action" of atropine is not of the same nature as the ac. ch.-antagonizing effect; it cannot be explained as an antagonizing of ac. ch. liberated at the stimulation of the motor nerve.

As early as 1903 CUSHNY emphasised an essential difference between the vagomimetic and the "curare-like" action of atropine. On various organs he studied the action of the two optical isomeres of hyoscyamine, which are the constituents of atropine. He found that the parasympathetic effect of atropine was most probably entirely due to its content of l-hyoscyamine. On the parasympathetic innervation of iris, salivary glands, and heart l-hyoscyamine was found to be twice as effective as atropine and 12—15 times as effective as d-hyoscyamine. LAIDLAW (1909), probably

using a purer preparation of d-hyoscyamine, found a still greater difference. As to the curare-like effect, however, CUSHNY found d-hyoscyamine to be quite as effective as l-hyoscyamine. For completeness, I have measured the antagonistic power of both isomeres (delivered by BURROUGHS and WELLCOME) on the action of ac. ch. on heart as well as on m. rectus abdominis of frog. In both cases l-hyoscyamine was found to be 30—40 times as effective as the dextrogyre isomere. Together with the findings of CUSHNY, this shows that the "curare-action" is not of the same nature as the ac. ch.-antagonizing, vagomimetic effect of atropine.

This conclusion is also supported by the fact that the direct excitability of the muscles is suppressed by atropine, and, according to DALE and his co-workers the direct excitability has nothing to do with the liberation of ac. ch. The long period of latency also speaks in the same direction. In case of atropine sulphate 1 : 75 the time of latency was found to be more than two hours. As shown by fig. 1, also the ac. ch.-antagonistic effect has a considerable period of latency before a certain amount of atropine has developed its full action, but on the other hand the greatest part of the antagonistic effect appears within about 15 minutes.

Thus it must be said that the effect of ac. ch. added to a striated muscle can be abolished by atropine, while even a massive concentration of atropine does not exert any effect on the indirect excitability of skeletal muscles which can reasonably be explained as an antagonizing of ac. ch. This discrepancy scarcely accords with the theory of ac. ch. as the humoral transmitter at the myoneural junctions of skeletal muscles.

DALE and his co-workers are of opinion that this discrepancy cannot be considered to have any decisive significance to the question of ac. ch. as myoneural transmitter. They point out that all parasympathetic nerves most probably have the same transmitter and this transmitter is most probably ac. ch. They refer to statements in the literature that in certain organs the effects of stimulation of parasympathetic nerves cannot be antagonized by atropine, which, however, antagonizes the effect of added ac. ch. According to their opinion, ac. ch. may be the transmitter at the myoneural junctions of skeletal muscles, although atropine exerts no specific action on the indirect excitability of striated muscles.

Against this argumentation may be said that in reality atropine suppresses all effects of stimulating parasympathetic nerves, even

if in some cases it is necessary to use rather large amounts. As CUSHNY (1924) points out, most observers have only studied the influence of smaller amounts of atropine, and therefore it is often stated that atropine has no influence on the effects of stimulation of the following parasympathetic nerves: vasodilatating nerves of chorda lingualis, motor innervation of stomach, intestine, urinary bladder, and of uterus. They are, however, paralyzed by larger amounts of atropine. As early as 1895 LANGLEY and ANDERSON found that about 10 mg per kilo of atropine paralyzed the motor innervation of rabbit's intestine, and in 1896 the same authors showed that atropine paralyzed the motor innervation of the urinary bladder. They have been confirmed by later authors. Recently HARRISON and McSWINEY (1936) found the motor innervation of stomach to be paralyzed. Just as is the case with added ac. ch., the amounts of atropine necessary for abolishing the effects of parasympathetic nerves in various organs vary within rather wide limits. V. E. HENDERSON (1923) measured the quantities of atropine necessary for paralyzing various parasympathetic nerves, which he found to be influenced by increasing concentrations of atropine in the following order: heart vagus, glandular nerves of chorda lingualis, vasodilatating nerves of chorda lingualis, and motor nerve of intestine; the parasympathetic nerves of uterus were not quite suppressed by the amounts used by HENDERSON.

It must, therefore, be said that the theory of humoral transmission at present offers no acceptable explanation of the discrepancy between the influence of atropine on the ac. ch.-effect and on the indirect excitability of skeletal muscles.

Summary.

1) DALE's original definition of the different actions of ac. ch. stated that muscarinelike actions are those which are abolished by atropine, while the nicotineline actions are not antagonized. Later experiments show that both kinds of actions are antagonized by atropine, even if several exceptions from this rule are described. There is, however, a quantitative difference between muscarine-like and nicotineline actions with regard to the atropine antagonism. As a rule, muscarinelike actions are inhibited by smaller concentrations, while nicotineline actions require higher concentrations of atropine. This quantitative difference is by some authors looked upon as a criterion of a fundamental distinction

between the two kinds of action. The nicotinelike actions require, however, not only larger amounts of atropine, but also large amounts of ac. ch. It seems possible that those properties of an organ which make large amounts of ac. ch. necessary also make it necessary to use large amounts of atropine before the antagonism can be demonstrated. It is shown in the present paper that there is a constant ratio between the amounts of atropine which are necessary to provoke a certain effect on various organs of frog and the amount of atropine which is necessary for diminishing this effect to a certain degree. Thus, the quantitative differences between the two kinds of action with regard to the atropine antagonism do not seem to constitute any real distinction.

2) Striped muscles of frog must be exposed to atropine during 50 to 60 minutes, before the atropine exerts its full antagonistic power.

3) It has been shown previously that atropine antagonizes the effect of ac. ch. on striped muscles, when these are suspended in a bath, while the effect of intraarterially injected ac. ch. is not abolished. In the latter case too small amounts of atropine seem to have been used. In the present paper it is shown that atropine prevents the "quick contractions" of skeletal muscles of frog or rabbit, provoked by intraarterial injections of ac. ch.

4) Those amounts of atropine which abolish the "quick contractions" do not have any influence on the excitability of the muscles through their nerves. If, however, the muscles are suspended in very strong concentrations of atropine they lose at first their indirect excitability and then also their direct excitability. Several reasons — *inter alia* the effect of d- and l-hyoscyamine — show that this so-called 'curare-action' of atropine is not of the same nature as the ac. ch.-antagonizing effects of atropine. Thus, while the effects of added ac. ch. are abolished, atropine exerts no action on the indirect excitability which can be explained as an antagonizing of ac. ch. It is emphasized that the theory of humoral transmission at present offers no acceptable explanation of this discrepancy.

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Rate of Penetration of Phosphate into Muscle Cells.¹

By

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(With 1 fig. in the text.)

Certain constituents of the voluntary muscle are able to diffuse out of the muscle into a surrounding saline and can also diffuse into the muscle if previously dissolved in the saline in a sufficiently high concentration. There exists, in such cases, a given concentration of the substance in saline which will be in equilibrium with the tissue. This critical concentration provides a measure for the concentration of the substance in the tissue — or rather in such part of the tissue as is concerned in the diffusion. M. G. EGGLETON (1933) carried out such experiments² in respect of phosphate exchange by immersing in Ringer's fluid an excised frog muscle at 2° for a few hours and found that in the resting muscle 20—30 per cent of the muscle water, corresponding to about 16—24 per cent of the weight of the fresh muscle, was involved in the diffusion system. Since 8—16 per cent of the weight of the gastrocnemius is composed of the interspaces into which the phosphate of Ringer's fluid will easily penetrate, the result mentioned above suggests either that phosphate can diffuse only into a certain fraction of the tissue beyond the extracellular volume or else that phosphate ions can penetrate only slowly into the muscle cells. That the latter alternative is more likely follows from the fact that no perceptible decrease in the total acid soluble phosphate content of muscles is apparent after fatigue, though a very perceptible increase in the inorganic P content

¹ Received 5 September 1940.

² Comp. also STELLA (1928).

of such muscles takes place. If the cell membranes were easily permeable to phosphate ions, a part of this excess phosphate should soon leak out into the plasma.

The application of the method of isotopic indicators permits us to follow the path of the labelled phosphate ions introduced into the circulation by making use of radioactive measurements. Due to the great sensitivity of this method it is possible to determine even very small amounts of labelled phosphate ions which migrate under strictly physiological conditions into the muscle cells during a few hours or less.

Description of the method.

Sodium phosphate of negligible weight containing radioactive P as an indicator is introduced into the circulation of the frog (injected into the lymph sack). After the lapse of, for example, ten hours, we compare the labelled phosphorus (^{32}P) content of a plasma sample and of a gastrocnemius sample of the same weight by determining their radioactivity. Let us assume that 1 gm plasma is found to be 5 times more active than 1 gm muscle tissue and the interspaces to make up $\frac{1}{10}$ of the muscle's weight, then the amount of phosphate ions which penetrated from 1 gm plasma into the cells of 1 gm muscle works out to be $\frac{1}{10}$ of that present

in 1 gm plasma or, in general, is $\frac{m}{p} - i p$, where m denotes the activity of 1 gm muscle, p the activity of 1 gm plasma, and i the size of the interspaces as a fraction of the muscle weight. When carrying out the calculation given above, we assume that ^{32}P becomes equally distributed between plasma and interspaces in an early stage of the experiment. How far this assumption is justified will be discussed later.

The experiment described above is carried out under strictly physiological conditions; the phosphorus content of the plasma and the muscle remains practically constant during the experiment and we can, therefore, conclude that the penetration of phosphate ions from the plasma into the muscle cells was followed by a migration of an equal number of phosphate ions from the muscle cells into the plasma. If an equilibrium is reached with an uptake of less ^{32}P than corresponding to the total water content of the system, we must assume saturation of a certain fraction of the tissue but, when the relative concentration of ^{32}P conti-

nuously increases in the muscle, we can utilize the results to measure the rate of exchange between cellular and extracellular phosphorus.

The application of the method outlined above requires the knowledge of the extent of the interspaces of the muscle tissue. The size of the interspaces can be obtained by comparing the chloride or sodium content of muscle and plasma samples of the same weight or by other methods. When applying the first mentioned method, the assumption is made that all sodium and chlorine present in the tissue is to be found in the interspaces. FENN and COBB (1935) state for the chlorine space of the sartorius of *rana pipiens* values varying between 7.5 and 16.0 per cent, the average being 11.3 per cent.

To determine the size of the extracellular space of the gastrocnemius of the Hungarian frog (*Rana esculenta*) used in our experiments, we administered labelled sodium along with the labelled phosphate. By measuring the distribution of the labelled sodium ^{24}Na between plasma and fresh gastrocnemius of equal weight we arrive at a figure indicating the extracellular volume of the muscle. The amount of extracellular phase (E) in percent of the weight of the muscle is calculated (MANERY and HASTINGS 1939) from the equation

$$E = \frac{(^{24}\text{Na})_m \cdot 0.97 \cdot 100}{(^{24}\text{Na})_p \cdot 0.99}$$

in which the subscripts m and p represent muscle and plasma, respectively¹.

The size of the interspaces being known, the measurement of the distribution of ^{32}P between plasma and muscle permits us, as described above, to determine the amount of ^{32}P which penetrates into the muscle cells.

A simultaneous measurement of the radioactivity of sodium and phosphorus is made possible by the fact that ^{24}Na decays with a half-life period of 14.8 hours while ^{32}P decays with a period of 14.5 days. When measuring the activity of the sample, for example, two weeks after the start of the experiment, the ^{24}Na

¹ When carrying out the calculation mentioned above we assume that the extracellular phase is identical with the ultrafiltrate of serum. The water content of the extracellular phase is assumed to be 99, that of the plasma 95 per cent. We arrive at the figure 0.97 by taking into consideration that the sodium ion concentration of the plasma and its ultrafiltrate somewhat differs and by calculating the difference from the Gibbs-Donnan equation.

originally present in the tissue and the plasma is entirely decayed and the activity measured is solely due to the ^{32}P content. Let us say we measured at that date 10 counts per minute, then two weeks previously the activity of the ^{32}P of the preparation was 20 counts. Assuming we measured, two weeks previously, a total of 100 counts, then out of these 80 counts were due to the ^{24}Na content and 20 counts to the ^{32}P content of the gastrocnemius sample. The accuracy of the determination can be augmented by administering a preparation which is showing a strong ^{24}Na and a comparatively weak ^{32}P activity. In view of the variability of the size of the interspaces in different muscles and in different frogs (comp. EGGLETON *et alia* 1937), it can be of importance to determine the extracellular volume of the muscle the permeability of which to phosphate ions is to be determined.

In experiments of short duration, the P activity of the plasma is solely due to the presence of radioactive inorganic P, the amount of radioactive phosphatides present being negligible and the plasma containing but an insignificant amount of acid soluble organic P. In experiments of very long duration, we are not permitted to use the total activity of the plasma, but we have to extract the plasma inorganic P and to compare its activity with the total activity of the muscle cells, assuming that only inorganic P can penetrate into the cells and is afterwards largely combined in the cells.

Results.

The distribution of ^{24}Na and of ^{32}P between equal weights of plasma and gastrocnemius muscle is seen in Table 1. The ^{24}Na was administered as 0.6 per cent sodium chloride solution which contained a negligible amount of active sodium phosphate. The solution was injected into the lymph sack of the frog. While, in experiments taking no more than two hours, the phosphate solution was administered at the start of the experiment, in experiments of longer duration a steadily decreasing volume of the labelled P solution was injected at intervals of two hours all through the experiment. Due to the uptake of the labelled phosphorus by bone and other tissue, the ^{32}P concentration of the plasma strongly decreases during an experiment unless kept up in this way. In the case of sodium which is not taken up by the cells to any appreciable extent, the ^{24}Na concentration of the plasma does not much decrease with time.

In Table 1 it is seen that, while the apparent sodium space makes out 4 per cent only of the weight of the muscle after the lapse of 3 min., after 20 minutes 14 per cent are found, which almost corresponds to the actual sodium space. The fact that between 20 minutes and 4 days the ratio of the ^{24}Na content of plasma and muscle of equal weight hardly changes is showing clearly that no significant uptake of 2 ^{24}Na by the muscle cells takes place in the course of 4 days.

Table 1.

Distribution of ^{24}Na and ^{32}P , respectively, between plasma and gastrocnemius¹ of equal weight at 22°.

Time after administration of the ^{24}Na and ^{32}P	$\frac{^{24}\text{Na}_m}{^{24}\text{Na}_p}$	$\frac{^{32}\text{P}_m}{^{32}\text{P}_p}$
3 min.	0.046	0.015
9 min.	0.12	—
20 min.	0.14	—
1 hour	—	0.155
10 hours	0.15	0.29
2 days	—	1.19
4 days	0.14	2.47

The subscripts m and p represent muscle and plasma, respectively.

The amount of ^{32}P which penetrates into the muscle tissue in the course of the first 3 minutes is much smaller than that of ^{24}Na ; this phenomenon is due to a slower diffusion of the phosphate ions through the capillary wall. After the lapse of 1 hour, a slightly greater percentage of the plasma ^{32}P is found in the muscle tissue than of the plasma ^{24}Na , the difference increasing with time. This phenomenon is due to a successive penetration of ^{32}P into the muscle cells. The amount migrated into the cells is obtained by subtracting the sodium space from the *apparent* phosphorus space. After 10 hours we find, as seen in Table 1 and Fig. 1, that the amount of ^{32}P which diffused into the cells of 1 gm gastrocnemius makes up 14 per cent of the ^{32}P content of 1 gm plasma or about 100 per cent of the ^{32}P present in the interspaces of 1 gm gastrocnemius. After the lapse of 4 days, the corresponding figures are 233 and 1653 per cent, respectively. The rate of penetration

¹ The extracellular volume in per cent of the muscle weight is obtained by multiplying the above figures by 98 (see p. 173).

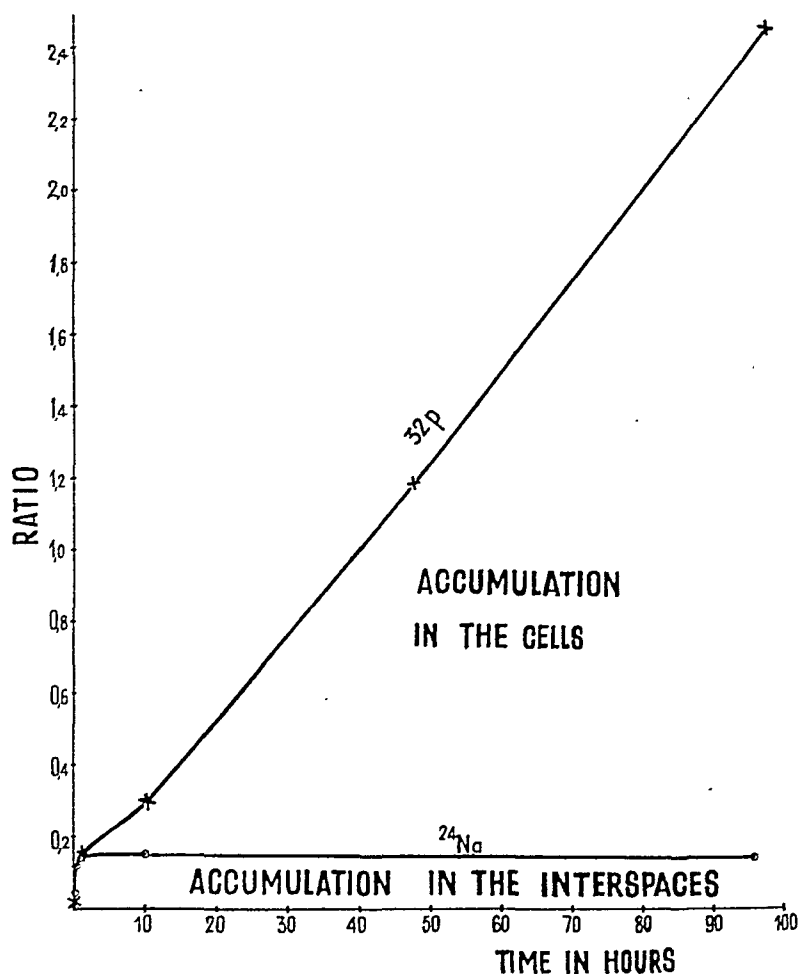


Fig. 1.

of phosphate ions into the muscle cells of the frog is, thus, a very slow one even at 22° and still slower at lower temperature. After the lapse of 10 hours at 0° , the *apparent* phosphorus space of the gastrocnemius was found to be 18 per cent; thus, only about $\frac{1}{5}$ as much labelled phosphate diffused into the cells at 0° than at 22° .

As mentioned above, we arrive at the values stated for the amount of labelled phosphate which migrated into the muscle cells by subtracting from the total amount of ^{32}P found in the muscle the amount of ^{32}P present in the interspaces. The accuracy of the figures obtained depends largely upon the accuracy of the figure assumed for the size of the interspaces.

In determining the extracellular volume we make two assump-

tions: a) We assume that all sodium or chlorine present in the muscle cells is exclusively found in the interspaces; b) we assume the concentration of sodium and chlorine, respectively, to be the same in the plasma water and the extracellular fluid. Much evidence is available that these assumptions are essentially correct. It is possible, however, that a small amount of sodium or chlorine penetrates into the cells (HASTINGS and EICHELBERGER 1937) and also that the extracellular fluid does not show exactly the same sodium or chlorine content as the plasma (comp. MANERY et alia 1938). The size of the interspaces calculated from the distribution figures of sodium and chlorine, respectively, is, however, about the same. FENN and COBB (1936) found the average sodium space and the average chlorine space of the rats' muscle to be 12.6 and 11.4 per cent, respectively. MANERY and HASTINGS (1939) state the apparent extracellular space of the gastrocnemius of the rabbit to be 11.3 per cent, calculated from the distribution of chlorine, and 11.0 per cent from the distribution of sodium, while for the abdominal muscle they give the figures 16.3 and 13.9 per cent, respectively.

We assume, furthermore, that an equal distribution of the labelled phosphate between plasma and extracellular fluid takes place at an early stage in the experiment. This assumption involves some uncertainty. After the lapse of 3 minutes (see Table 1), an equal distribution is far from being reached by either sodium or phosphate; after 1 hour equality may be reached also by the phosphate, but the possibility cannot be excluded that the equality of the sodium space and the phosphate space found after the lapse of 1 hour is a fortuitous one and is due to the fact that some ^{32}P penetrates into the cells before the equipartition mentioned above was obtained, the sum of the cellular and extracellular P present in 1 gm muscle making out 14 per cent of the ^{32}P content of 1 gm plasma. In the case of the brain tissue, the capillaries of which are only at a slow rate permeable to phosphate, we found obvious indications of a penetration of ^{32}P into the cells before an equipartition of ^{32}P between plasma and extracellular fluid was obtained. It is, therefore, of importance to find a method which permits us to determine the amount of ^{32}P penetrating into the cells without having to make any assumption regarding the size of the interspaces and the time involved in obtaining an equal distribution of ^{32}P between plasma and extracellular fluid. Such a method will be described in the following section.

Description of the modified method.

When applying the modified method, we compare the activity of the inorganic P of a plasma sample of known weight with the activity of the *organic* phosphorus extracted from the muscle sample of the same weight. This method is based on the assumption that the organic phosphorus compounds present in the muscle tissue are formed in the muscle cells from inorganic phosphate and that, correspondingly, all active P atoms present in the organic constituents of the muscle are such which passed from the plasma into the cells as inorganic ^{32}P . Since some of the active phosphate penetrated into the cells will not have had opportunity to be incorporated into organic molecules, but will remain in inorganic state, the method here outlined will give a lower limit for the extent of phosphorus exchange between plasma and muscle cells. By adding to the activity of the organic P of the muscles that of the cellular inorganic P we arrive at the total cellular activity.

In experiments of several hours' duration or more, we can estimate the amount of cellular inorganic ^{32}P by the following consideration: Let us consider an experiment taking 10 hours. We find that, in this experiment, 66 per cent of the ^{32}P content of the acid soluble organic P of the muscle is present as creatine-phosphoric acid P and that the amount of creatinephosphoric P makes out 2.4 times that of the inorganic P present as such in the muscle. The last mentioned data are obtained by the usual chemical determination of creatine P and inorganic P, respectively. Since from activity data we know that, in the course of 10 hours, almost all the creatinephosphate molecules present in the muscle get renewed, the ^{32}P content of 1 mgm inorganic P will be about equal to the ^{32}P content of 1 mgm creatine P. From these data it follows that the activity of the cellular inorganic P makes out $66 : 2.4 = 28$ per cent of that of the cellular acid soluble organic P. We have, thus, to add to the values obtained for the acid soluble organic cellular ^{32}P content of the muscle (see Table 3, column 2) 0.28 times the value obtained in order to get the value of the total ^{32}P migrated into the muscle cells during 10 hours. In an analogous way the other figures seen in column 2 of Table 3 were obtained. The correction due to the presence of ^{32}P in the cellular inorganic P fraction was smaller in experiments of longer duration.

In the consideration stated above we have disregarded the fact that the amount of inorganic P extracted from the muscle is partly

extracellular P. This procedure is permissible when dealing, as above, with chemical magnitudes alone in view of the fact that the amount of cellular inorganic P is about 60 times larger than that of the extracellular inorganic P of the muscle. We meet, however, very different conditions when considering the radioactivity of the cellular and extracellular inorganic P, respectively, (which is not the case in the consideration made above). Due to the slow migration of the phosphate ion into the muscle cells, the activity of 1 mgm extracellular inorganic P may be many hundred times larger than that of 1 mgm cellular inorganic P.

Table 2.

Distribution of ^{32}P between the acid soluble organic constituents extracted from 1 gm gastrocnemius and the inorganic phosphate extracted from 1 gm plasma.

Time after administration of ^{32}P	Distribution coefficient	Percentage ¹ of organic acid soluble P replaced by plasma P
10 hours	0.11	0.5
2 days	1.02	2.4
4 days	2.11	8.0

Table 2 contains data on the ratio of the activity of the inorganic P of plasma samples and that of the acid soluble organic P fractions isolated from muscle samples having the same weight as the plasma samples. These ratios are stated in column 2 and indicate, as mentioned above, the lower limit of the fraction of the plasma P which exchanged with cellular acid soluble P during the experiment. Column 3 contains data on the percentage of the organic acid soluble P which was replaced by plasma P during the experiment. In the course of 4 days, 8 per cent was replaced.

The amount of ^{32}P incorporated into the non acid soluble P of the frogs' muscle is very restricted. After the lapse of 4 days, the number of ^{32}P atoms incorporated into phosphatides makes up 2.5 per cent of the amount present in the acid soluble compounds. For the ^{32}P incorporated into residual (protein) P, the corresponding figure was found to be 2.6 per cent. Thus, the amount of ^{32}P

¹ The ratio of the acid soluble P content of 1 gm muscle and 1 gm plasma was found in the frog killed after 10 hours, 2 days and 4 days to be 31, 42 and 26.4 respectively. The variation of the above ratio is to a large extent due to a variation in the acid soluble P content of the plasma which was found to be 3.6, 3.6 and 4.8 mgm per cent respectively.

incorporated into all phosphorus fractions present in the muscle is, after the lapse of 4 days, but 5 per cent larger than the amount incorporated into the acid soluble compounds. In experiments of shorter duration the difference is still less.

Table 3.

Distribution of ^{32}P between the total cellular acid soluble phosphate extracted from 1 gm gastrocnemius and the inorganic phosphate extracted from 1 gm plasma.

Time after administration	Distribution coefficient obtained by the modified method (organic fraction extracted, share of inorganic P computed)	Distribution coefficient obtained by the original method (direct comparison of the activity of muscle and plasma of equal weights after subtraction of ^{32}P content of the extracellular volume)
10 hours	0.14	0.14
2 days	1.20	1.05
4 days	2.50	2.33

In Table 3, the distribution coefficient of ^{32}P between cellular P and plasma P arrived at by the two different methods is given. Column 2 contains the distribution coefficient calculated from the ^{32}P content of the organic fractions and of the inorganic phosphate content of the muscle, as described on p. 178, while in column 3 the results are given which were obtained by comparing the activity of the muscle tissue with that of the plasma. The results obtained agree fairly well.

It is of interest to remark that the labelled phosphate was found to penetrate at a faster rate into the cells of mammalian muscle than in those of the frog (Hahn et alia 1939). The ratio of the ^{32}P content of 1 gm gastrocnemius and of 1 gm plasma of the rabbit was found after the lapse of 4 hours to be 0.6.

Discussion.

The concentration of the inorganic phosphate present in the water of the muscle cells is about 100 times larger than that present in the plasma water. This puzzling difference can be explained in two different ways. We can assume that most of the inorganic phosphorus extracted after a most careful treat-

ment of the muscle was not present as inorganic phosphate previous to extraction in the muscle but in the form of a very labile phosphorus compound. Creatinephosphoric acid is a fairly labile compound which can only be extracted without decomposition if the operation is carried out at a low temperature and a very fast rate. It is quite conceivable that a decomposition of other still more labile phosphorus compounds during extraction cannot be avoided. But the existence of the great difference in the inorganic phosphate concentration of plasma and cell water can be explained in a very different way as well.

The muscle cells take up when formed a comparatively large amount of inorganic P. This high inorganic P content is maintained all through life, the cell walls being impermeable to phosphate ions or, as far as a restricted permeability is present, the phosphate lost by the cells is compensated by a secretion of an equal amount of phosphate from the plasma into the muscle cells. Numerous examples of such active secretion are reported by KROGH (1939), and the application of his views to the present problem leads to the last mentioned explanation.

It is not possible at present to decide which of the explanations mentioned above is the correct one; the application of radioactive P as an indicator leads, however, to the result that a restricted permeability of the muscle cell wall to phosphate ions is actually present. Professor KROGH has kindly drawn our attention to a possibility of deciding which of these explanations is the right one. According to his view, the primary process is the loss of some cellular phosphate by leakage through the cell wall. The extent of the active secretion into the cells is that necessary to compensate for the loss by leakage and is determined by the extent of the latter. Let us increase the phosphate concentration of the plasma by administering large amounts of phosphate for example. This increase should, according to the view cited above, not influence the amount of labelled phosphate penetrating into the cells while, in the case that the entrance of labelled phosphate into the cells is due to diffusion, the amount entering the cells from a plasma containing more phosphate should be larger than from one containing less.

We wish to express our hearty thanks to Professor NIELS BOHR and Professor AUGUST KROGH for numerous facilities most kindly put at our disposal.

Summary.

Labelled sodium and labelled phosphate are injected simultaneously into the lymph sack of the frog and the distribution of the radioactive sodium and the radioactive phosphate between plasma and muscle of equal weights is determined. A constant partition ratio of the radioactive sodium is obtained after the lapse of about 20 minutes. From this ratio the volume of the interspaces of the muscle can be calculated. In the case of phosphorus, the partition ratio increases even after the lapse of many days because of continued penetration of the labelled phosphate into the cells. The difference of the partition ratio of the radioactive phosphorus and the radioactive sodium permits us to calculate the amount of ^{32}P which penetrated into the muscle cells. Since all phosphorus atoms present in the plasma can be assumed to show the same behaviour as the ^{32}P atoms we can compute from the figures obtained the amount of plasma inorganic P which exchanged with cellular P during the experiment. In the course of 4 days at 22° , 0.082 mgm P was found to penetrate into the cells of 1 gm gastrocnemius muscle and vice versa. At 0° , $\frac{1}{5}$ of the above value was found.

An alternative method which is independent of the knowledge of the size of the extracellular space is based on the determination of the comparison of the active inorganic phosphorus content of the plasma with the active *organic* phosphorus content of the muscle. When applying this method, one assumes that the organic phosphorus compounds of the muscle are renewed inside the cells. By this method, the amount of plasma P penetrated into 1 gm muscle in the course of 4 days was found to be 0.088 mgm.

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Über den Einfluss lokaler physikalischer und chemischer Hautreize auf die periphere Blutverteilung.¹

Von

GÖSTA von REIS und FRITIOF SJÖSTRAND.

(Mit 4 Figuren im Text.)

In früheren Arbeiten (G. von REIS und F. SJÖSTRAND 1937, 1938) wurden die Resultate einer Untersuchung über den Einfluss von lokaler Hautreizung mittels Senföl und Ultraviolettstrahlung auf die periphere Blutverteilung in der Leber und Nierenrinde bekanntgegeben. Mit beiden Reizmitteln wurde eine erhebliche Zunahme der peripheren Blutmenge in diesen Organen erzielt, ein Effekt, welcher ausblieb, wenn der gereizte Hautbezirk vorher denerviert worden war. Die vorliegende Arbeit enthält einerseits die Ergebnisse einer Untersuchung über die Wirkung lokaler thermischer Hautreizung auf die periphere Blutmenge in der Leber und Nierenrinde sowie auf die Anzahl offener Kapillaren im M. masseter, andererseits die Resultate von Bestimmungen der Anzahl offener Kapillaren im M. masseter bei gelegentlich der früher beschriebenen Versuche mit lokaler Hautreizung mittels Senföl und Ultraviolettstrahlung verwendeten Versuchstieren.

In früheren Studien über das Verhalten von Blutgefäßen bei verschiedenen thermischen Hautreizungen gestatteten die Methoden keine differenzierte Untersuchung der peripheren Blutgefäße (mit diesem Begriff sind hier Kapillaren, Sinusoide und sinuöse Blutgefäße gemeint). Eine Ausnahme stellen die Untersuchungen von T. SJÖSTRAND (1934, 1935) dar, welche sich jedoch auf den Einfluss verschiedener Lufttemperaturen, also auf generelle Kälte- oder Wärmereize beziehen.

¹ Der Redaktion am 14. September 1940 zugegangen.

Dieser Autor untersuchte mit derselben Methode wie wir bei Mäusen die Blutmenge in peripheren Blutgefäßen der Nebennieren, Leber, Nieren und Skelettmuskulatur, nachdem die Tiere $1\frac{1}{2}$ —3 Stunden in einer Kammer mit einer Lufttemperatur von entweder $+1^{\circ}$ — $+6^{\circ}$ C oder $+35^{\circ}$ — $+37^{\circ}$ C geweiht hatten. Er erhielt dabei eine Zunahme der peripheren Blutmenge in den Nebennieren, der Leber und der Skelettmuskulatur bei Kälte, in der Leber und den Nieren bei Wärme.

Sonstige Untersuchungen auf diesem Gebiet sind meistens mit Hilfe von Plethysmographie, Kalorimetrie oder Thermostromuhr vorgenommen worden. Hierbei wurden die Reaktionen in Arterien, peripheren Blutgefäßen und Venen gleichzeitig registriert, und ein differenziertes Studium der Reaktionen der einzelnen Blutgefäßstypen war nicht möglich.

Dass eine lokale thermische Hautreizung auf Blutgefäße einwirken kann, welche von dem gereizten Hautgebiet mehr oder weniger weit entfernt sind, zeigt schon der Versuch von BROWN-SEQUARD und THOLOZAN (1858) über die sog. konsensuelle Innervation. Die Beobachtungen dieser Forscher sind später durch eine ganze Reihe von plethysmographischen Untersuchungen (A. MOSSO 1874, FRANÇOIS-FRANCK 1876, U. MOSSO 1889, AMITIN 1897, HEWLETT, VAN ZWALUWENBURG und MARSHALL 1911 und PICKERING 1931) sowie bei kalorimetrischen Bestimmungen von STEWART (1911) bestätigt worden.

Was u. a. die Hautgefäße betrifft, so sollen durch derartige Reflexe nicht nur symmetrisch liegende Hautpartien von einem lokalen thermischen Hautreiz in Mitleidenschaft gezogen sondern eine allgemeinere Reaktion in diesen Gefäßen ausgelöst werden (O. MÜLLER 1904, PICKERING 1931, FREEMAN 1935).

Bei Kaninchen fand WERTHEIMER (1894) nach Kurarisierung und Laparotomie, dass bei Abkühlung der Haut der arterielle Blutdruck stieg, während der Druck in der Vena renalis niedriger wurde. Nach Ansicht des Autors beruht der Effekt auf einer Kontraktion der kleineren Blutgefäße in der Niere mit daraus folgendem gesteigertem peripherem Widerstand. Nach Denervierung der Niere ging der Blutdruck in der Arterie und Vene vollkommen Hand in Hand.

Mittels Thermostromuhr konstatierte REIN (1929) am Hund, dass Kältereizung der Haut mit Eis in der Nähe der Nasenflügel eine Steigerung des Blutstroms in der Art. carotis comm. um 40 % mitsichbrachte. Bei genereller Abkühlung und während zweck-

mässig gewählter Narkose fand der gleiche Autor (1931) eine Zunahme der Durchströmung der Art. carotis comm. mit Blut von im Mittel 28 %, in der Vena renalis von 13 % und in der Vena mesenterica cranialis von über 400 %, und REIN und RÖSSLER (1929) stellten fest, dass die Durchblutung von Vena porta und Nierengefässen um 50—200 % stieg.

RUHMANN (1927) fand bei laparoskopischen Beobachtungen der grösseren Darmgefässe beim Menschen, dass Wärme dieselben zur Dilatation und Kälte zur Kontraktion bringt. Da der Effekt rasch eintritt meint der Autor, dass es sich um einen Reflex handle, welcher von der Reaktion in den Hautgefässen ausgelöst werden soll.

In späterer Zeit hat man sich neben dieser Reflexeinwirkung die Möglichkeit einer hormonal vermittelten Fernwirkung gedacht (LAQUEUR 1930, BORNSTEIN 1931, HOFF 1931).

Methodik.

Als Versuchstiere wurden 400—600 g wiegende Meerschweinchen verwendet.

Die Bestimmung der Blutmenge in peripheren Blutgefässen wurde mit der von T. SJÖSTRAND (1934) angegebenen Methode ausgeführt, ebenso wie bei unserer früheren Arbeit, weshalb wir auf diese verweisen (G. VON REIS und F. SJÖSTRAND 1938). Die bisher untersuchten Organe sind die Leber, Nierenrinde und ein Skelettmuskel, der M. masseter. Bei der Feststellung der peripheren Blutmenge in der Nierenrinde wurde nur die Blutmenge der rings um die Tubuli liegenden Blutgefässe und nicht die der Glomeruli bestimmt.

Auch sonst stimmt das Verfahren mit dem bei unseren früher beschriebenen Versuchen in Anwendung gebrachten überein.

Der gereizte Hautbezirk bestand aus einem 3×6 bis 4×6 cm grossen Gebiet quer über die Bauchhaut. Dieses Hautgebiet war am Tage vor Ausführung des Versuchs rasiert worden. Der Hautreiz wurde unter Pernoctonnarkose appliziert. Die Behandlung wurde an in Rückenlage festgeschnallten Tieren vorgenommen und dauerte in der Regel 45 Min. Nach der Behandlung wurden die Tiere durch Abquetschung des Halsmarks oder durch Dekapitation unmittelbar getötet. Bei Versuchen mit Reizung von denervierter Haut war die Nervendurchtrennung 10—20 Tage vor dem Versuch ausgeführt worden.

Der Kältereiz wurde mittels eines Gummischlauchs appliziert, durch welchen kaltes Wasser geleitet wurde.

Zur Wärmereizung wurden teils ähnliche Verfahren mit warmem Wasser, teils eine Wärmelampe (»Glory«) verwendet, welche Wärmestrahlen von sowohl grösserer wie geringerer Wellenlänge aussendet und in verschiedenen Abständen von den Tieren angebracht wurde.

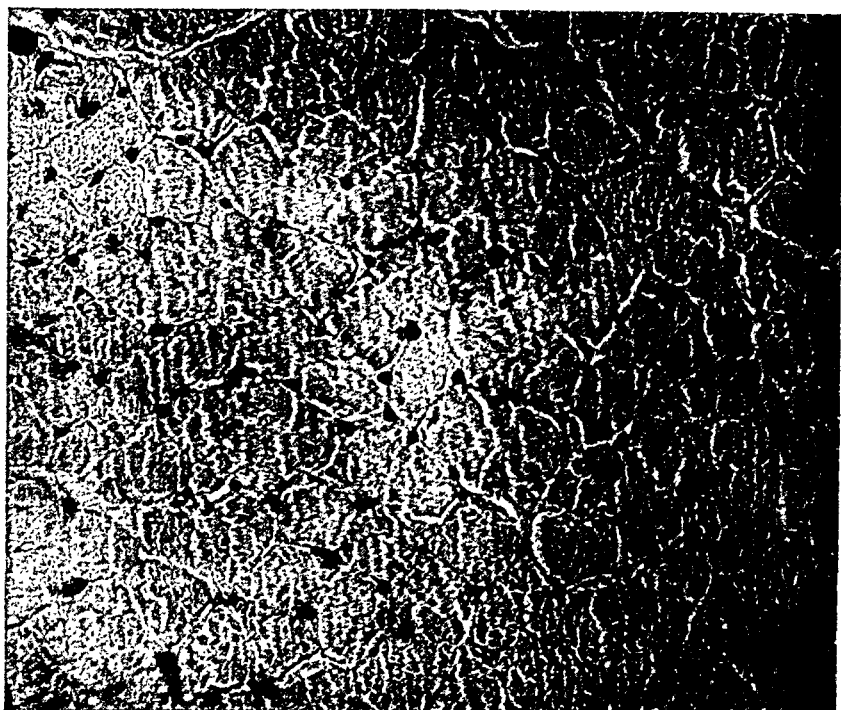


Abb. 1. Querschnitt durch den M. masseter. Die Muskelkapillaren werden punktförmig projiziert. Schnittdicke: $20\ \mu$. Färbung: Ortho-Tolidin-wasserstoffsuperoxyd.

Bei Versuchen mit der Wärmelampe wurde die Wärmestrahlung mittels eines zweckmässig geformten, mit Wasser gefüllten Blechgefässes begrenzt. Die Lufttemperatur in der Umgebung des Tieres unter diesem Gefäss stieg dabei um ungefähr 1°C .

Bei der Wärmebestrahlung wurde die Hauttemperatur innerhalb des behandelten Gebiets mit einem Thermoelement (Konstantan-Kupfer) gemessen.

Bei der Bestimmung der Anzahl offener Kapillaren pro Flächeneinheit im Querschnitt des M. masseter wurden $20\ \mu$ dicke Querschnitte des Muskels verwendet. Nach Färbung der roten Blutkörperchen mit Ortho-Tolidinwasserstoffsuperoxyd wurde die Anzahl blutgefüllter Kapillaren durch Zählung unter dem Mikroskop bei ungefähr 540facher Vergrösserung bestimmt, wobei wir uns eines im Okular angebrachten Zählquadrats bedienten. Durch das Zählquadrat wurde von dem Muskelquerschnitt eine quadratische Fläche von $0.082\ \text{mm}^2$ abgegrenzt. Die Kapillarenanzahl wurde in 25 derartigen Quadraten von wenigstens 10 verschiedenen Schnitten und von verschiedenen Partien des Querschnitts gezählt.

Bei derartigen Bestimmungen ist es von grösster Bedeutung, dass die Zählungen an guten Querschnitten vorgenommen werden, da schräge Schnitte ja dazu führen müssen, dass die Schnittfläche einer



Abb. 2. Schrägschnitt durch den M. masseter. Die Kapillaren werden nicht punktförmig projiziert, sondern an den Blutkörperchen in der scharfeingestellten Ebene zeichnen sich die Blutkörperchen in den tieferen Partien des Schnitts diffus ab. Ein Unterschied in der Form des Muskelfaserquerschnitts gegenüber Abb. 1 wird trotzdem nicht sichtbar. Schnittdicke und Färbung wie Abb. 1.

jeden Muskelfaser grösser und damit die Kapillarenanzahl pro Flächeneinheit geringer wird.

So sinkt die Anzahl Kapillaren pro Flächeneinheit um 2 % bei einer Schnittschrägheit von 10° , um 15 % bei einer solchen von 30° und um 40 % bei 45° . Hieraus geht hervor, dass ein geringerer Grad von Schnittschrägheit keine grössere Fehlerquelle mitsichbringt, dass aber letztere schon bei mässigen Schrägheitsgraden höchst beträchtlich wird.

Um diese Fehlerquelle zu eliminieren wurden $20\ \mu$ dicke Schnitte verwendet, was von T. SJÖSTRAND empfohlen worden war. Wenn man nämlich mit einer Kapillarweite von ungefähr $2.5\ \mu$ rechnet, findet man, dass eine Schnittschrägheit von 15° zur Folge hat, dass die untere Grenzfläche einer quer durch den Schnitt verlaufenden Blutkörperchensäule ganze zwei Kapillardurchmesser im Verhältnis zur oberen Grenzfläche seitlich verschoben wird. Eine solche Verschiebung lässt sich natürlich bei scharfer Einstellung verschiedener Tiefen im Präparat leicht entdecken. Unsere Bestimmungen sind an Schnitten ausgeführt, wo der Schnittschrägheitswinkel maximal ungefähr 15° betrug.

Eine andere Möglichkeit, den Grad der Querschneidung zu beurteilen, bietet die Form der Schnittflächen der Muskelfasern. Aus mehreren

Gründen gestatten indessen diese nur sehr unsichere Schlussfolgerungen. So treten die einzelnen Muskelfasern, welche ungefärbt sind, nicht immer distinkt hervor und lassen sich oft nicht mit Sicherheit unterscheiden. Dadurch dass die Querschnitte der einzelnen Muskelfasern eine unregelmässige, abgerundet-kantige Form haben, kann die Schnittschrägheit einen erheblichen Grad erreichen, ohne dass eine deutliche Formänderung eintritt. Dies geht aus einem Vergleich der Abb. 1 und 2 hervor. Abb. 1 stellt einen guten Querschnitt dar, während Abb. 2 einen schräggesechnittenen Muskel zeigt, wo die Schnittschrägheit durch den Verlauf der Kapillaren deutlich markiert wird, wo man aber lediglich aus den Schnittflächen der Muskelfasern den Grad der Schrägheit nicht sicher entnehmen kann.

Da man an dünnen Schnitten den Grad der Schnittschrägheit nur durch Beobachtung der Schnittflächen der Muskelfasern beurteilen kann vermisst man daher bei diesen eine effektive Kontrolle des Schnittschrägheitswinkels; hierdurch wird eine erhebliche Fehlerquelle eingeführt, was nicht von allen beachtet worden zu sein scheint, welche sich dieser Methode bedient haben.

Aus verschiedenen Gründen wurden bei den Bestimmungen der Anzahl offener Kapillaren in der Muskulatur keine Methoden mit Vergleich mit Standardpräparaten oder Vergleich zwischen den einzelnen Präparaten verwendet. Einerseits sind nämlich die Variationen der Blutmenge und Dichte dieser Gefässe von einer erheblich geringeren Grössenordnung als es z. B. bei peripheren Blutgefässen in der Leber und Nierenrinde der Fall ist, weshalb die Differenzen weniger hervortreten, andererseits bieten die Muskelpreparate ein bunt zusammengewürfeltes Bild von miteinander abwechselnden Partien von Quer-, Schräg- und Längsschnitten, weshalb der visuelle Eindruck bei der für eine solche Beurteilung erforderlichen Übersicht des Präparats ein höchst uneinheitlicher wird.

Um einen gewissen Masstab für die Genauigkeit zu erhalten, welche bei den Einzelbestimmungen obwaltet, hat diejenige Person, welche durchweg die Bestimmungen bei sämtlichen hier veröffentlichten Serien ausgeführt hat, die Anzahl offener Kapillaren in zwei Muskeln bei drei bzw. vier verschiedenen Gelegenheiten mit mehreren Tagen Zwischenraum und ohne von den Doppelbestimmungen zu wissen bestimmt. Die Resultate der Bestimmungen gehen aus Tab. 1 hervor.

Diese Werte zeigen eine Variation zwischen den einzelnen Bestimmungen von in beiden Fällen maximal zwischen 6 und 7 %.

Die Anzahl offener Kapillaren pro mm² Querschnitt wurde in 11 Muskeln bei zwei verschiedenen Gelegenheiten bestimmt. Die Abweichung zwischen den beiden dabei erhaltenen Werten betrug für jeden einzelnen Muskel im Mittel 7.8 % \pm 1.08. Stellt man die 11 Werte, welche bei jeder Gelegenheit erhalten worden waren, zu zwei Serien zusammen und vergleicht man die Mittelwerte dieser Serien, so findet man eine Abweichung zwischen denselben von 1.8 %. Die Abweichungen zwischen bei verschiedenen Gelegenheiten ausgeführten Bestimmungen sind somit nicht systematisch, sondern gleichen bei genügend grossen Reihen einander aus.

Tab. 1.

Wiederholte Bestimmungen der Anzahl offener Kapillaren pro mm²
Querschnitt zweier Muskeln.

Bestimmung Nr.	M. masseter	
	Nr. 1	Nr. 2
1	1 680 ± 39	1 410 ± 41
2	1 630 ± 36	1 340 ± 53
3	1 580 ± 49	1 320 ± 42
4	1 600 ± 37	

Die statistischen Berechnungen wurden nach folgenden Formeln vorgenommen:

Bei Bestimmung der Anzahl offener Kapillaren in Querschnitten des M.masseter wurde die Streuung nach der Formel:

$$(1) \quad \sigma = \pm \sqrt{\frac{\sum a^2}{n-1}}$$

(a = Abweichung vom Mittelwert; n = Anzahl Beobachtungen, hier immer = 25, weshalb die diesbezügliche Korrektur angebracht wurde) berechnet.

Der mittlere Fehler des arithmetischen Mittels wurde nach der Formel:

$$(2) \quad \epsilon (M) = \pm \frac{\sigma}{\sqrt{n}}$$

bestimmt.

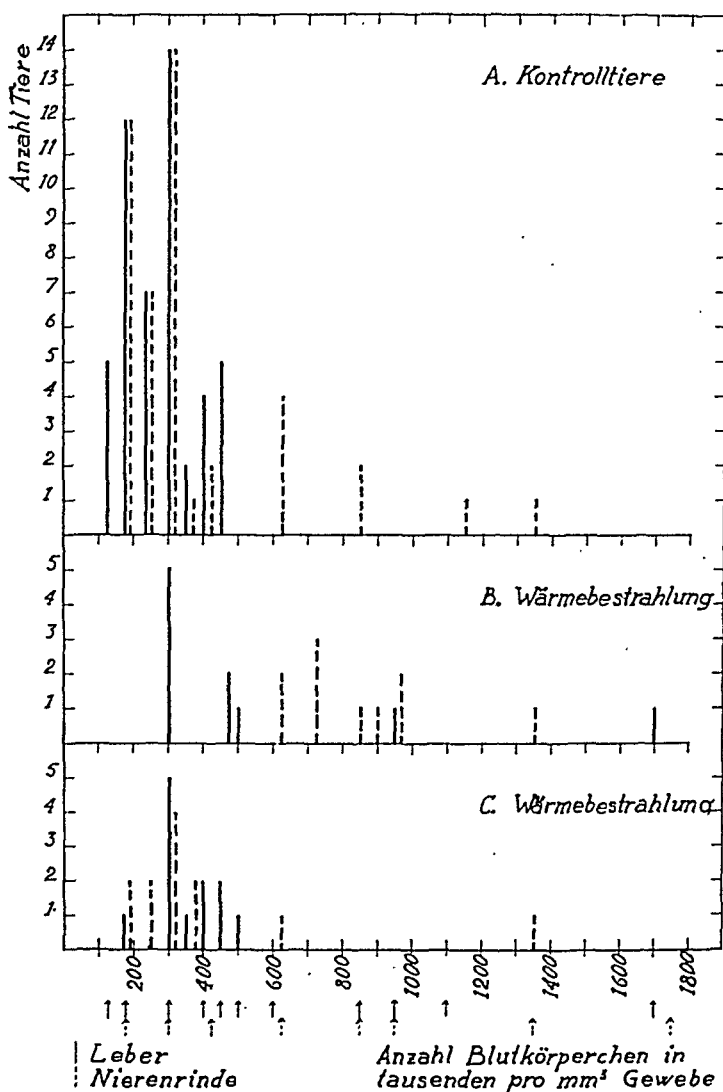
Bei dem Vergleich zwischen den Werten der verschiedenen Versuchsreihen fanden folgende Formeln Verwendung:

$$(3) \quad \sigma^2 = \frac{1}{m+n-2} \left[\sum^m (A_i - M_A)^2 + \sum^n (B_j - M_B)^2 \right]$$

$$(4) \quad \Sigma (M_A - M_B) = \sigma \sqrt{\frac{1}{m} + \frac{1}{n}}$$

$$(5) \quad t = \frac{M_A - M_B}{\Sigma (M_A - M_B)}$$

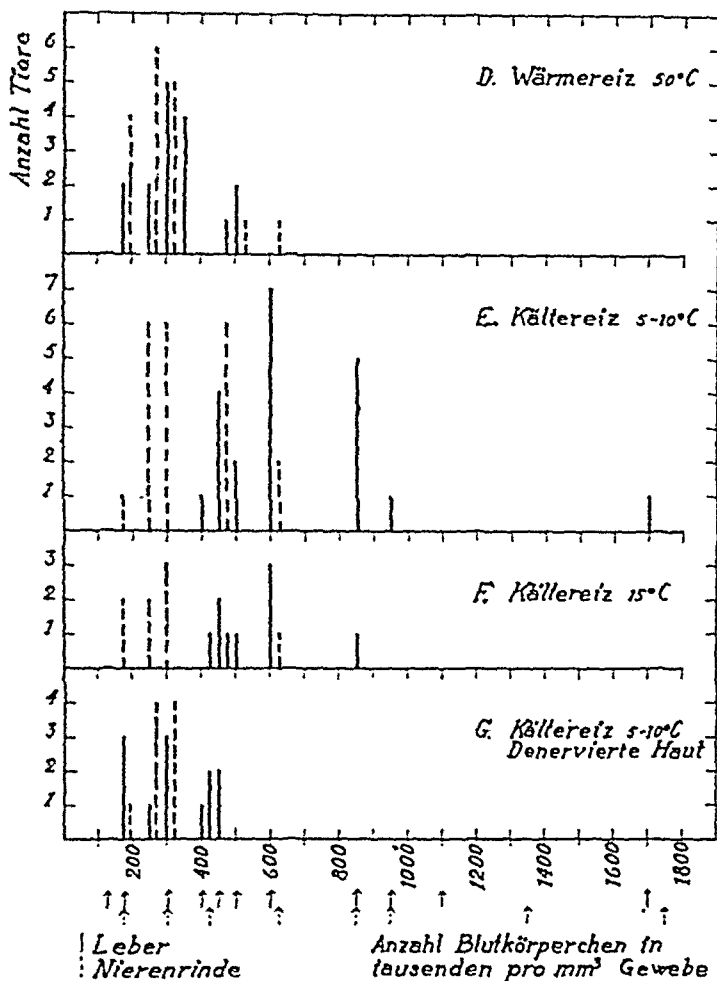
(m und n ist die Anzahl der Werte in den beiden Serien. A_i und B_j repräsentieren den einzelnen Wert in den betreffenden Serien. t stellt die Mittelwertsdifferenz dar, ausgedrückt in ihrem mittleren Fehler. Siehe »STUDENT« 1908 und WAHLUND 1931.)



Die Pfeile unter der Abszisse bezeichnen die Werte bei den verwendeten Standardpräparaten.

Diagramm 1.

Eine statistische Bearbeitung der bei Verwendung des Vergleichsstandards erhaltenen Werte wurde infolge der Schwierigkeiten, welche durch die unregelmässigen Intervalle der Standardpräparate verursacht werden, nicht vorgenommen. Eine derartige Bearbeitung erscheint auch im Hinblick auf die Grösse der Differenzen zwischen den Versuchsreihen überflüssig, welche hier in den meisten Fällen vorliegen. Stattdessen ist die Gruppierung der Primärwerte graphisch veranschaulicht.



Die Pfeile unter der Abszisse bezeichnen die Werte bei den verwendeten Standardpräparaten.

Diagramm 2.

V Versuchsergebnisse.

Kontrolltiere.

Gleichzeitig mit den in früheren Arbeiten beschriebenen Versuchen (Hautreizung mit Senföl und Ultraviolettstrahlung) wurden laufend Kontrolltiere untersucht. Im Laufe von reichlich einem Jahr erhielten wir ein grosses Kontrolltiermaterial, welches, obwohl die Tiere also in verschiedenen Jahreszeiten untersucht worden waren und aus verschiedenen Tierzüchtereien stammten, nicht solche Variationen hinsichtlich der peripheren Blutmenge aufwies, dass sie bei der Beurteilung der hier erhaltenen Resultate

Tab. 2.

Anzahl offener Kapillaren pro mm² Querschnitt des M. masseter.

Nr	Kontroll-tiere	Wärme-strahlung	Kontakt-wärme	Kälte	Senföl	Ultraviolett-strahlung
				intakte Haut		
1	1530 ± 54	1470 ± 45	1480 ± 49	1550 ± 54	1560 ± 50	1440 ± 55
2	1290 ± 40	1190 ± 26	1350 ± 50	1850 ± 64	1700 ± 34	1260 ± 40
3	1300 ± 46	1290 ± 42	1330 ± 42	1990 ± 37	1550 ± 44	1470 ± 35
4	1430 ± 63	1150 ± 38	1340 ± 44	1660 ± 43	1230 ± 38	1410 ± 28
5	1390 ± 57	1290 ± 39	1400 ± 30	1760 ± 60	1320 ± 39	1430 ± 49
6	1390 ± 51	1360 ± 37	1490 ± 36	1950 ± 55	1540 ± 62	1810 ± 39
7	1110 ± 46	1260 ± 36	1140 ± 34	1900 ± 51	1530 ± 52	1580 ± 51
8	1270 ± 48	1430 ± 37	1360 ± 41	1770 ± 41	1440 ± 51	1440 ± 52
9	1420 ± 52	1400 ± 43	M = 1360	1460 ± 49	1790 ± 37	1520 ± 39
10	1340 ± 53	1320		1610 ± 53	1510 ± 61	1690 ± 52
11	1460 ± 57			1760 ± 36	1680 ± 37	1660 ± 49
12	1250 ± 45			1480 ± 52	1190 ± 32	1360 ± 29
13	1220 ± 35			1660 ± 45	M = 1500	1740 ± 57
14	1330 ± 28			1630 ± 39		1340 ± 44
15	1310 ± 31			1700 ± 46		1580 ± 36
16	1330 ± 28			M = 1710		M = 1520
17	1390 ± 45					
18	1310 ± 43					
19	1290 ± 27					
M	M = 1330		denervierte Haut			
1				1370 ± 41	1210 ± 53	1410 ± 47
2				1280 ± 40	1390 ± 54	1370 ± 40
3				1370 ± 44	1220 ± 46	1480 ± 50
4				1350 ± 45	1180 ± 47	1220 ± 31
5				1440 ± 34	1150 ± 36	1420 ± 39
6				1360 ± 38	1310 ± 42	1140 ± 45
7				1420 ± 46	1350 ± 43	1190 ± 46
8				1360 ± 57	1230 ± 42	1230 ± 30
9				1270 ± 48	M = 1260	1210 ± 40
10				1600 ± 46		1450 ± 48
11				1440 ± 34		1560 ± 52
				M = 1390		M = 1330

ins Gewicht fallen könnten. Diese Kontrolltierwerte betrugen im Mittel für die Leber (49 Tiere) 268 000 und für die Nierenrinde (44 Tiere) 363 000 Blutkörperchen pro mm³ Gewebe. Diagramm 1 A zeigt die Gruppierung der Primärwerte.

Die Anzahl offener Kapillaren pro mm² Querschnitt im M. masseter wurde an 19 Kontrolltieren bestimmt. Der mittlere Wert der Bestimmungen beträgt 1 330. In Tab. 2 findet man die Primärwerte.

Diese Werte für Kontrolltiere liegen nicht unerheblich niedriger als die von T. SJÖSTRAND (1935) für Normaltiere und Tiere in Pernoctonnarkose angegebenen Werte, was ausschliesslich darauf beruht, dass verschiedene Prinzipien bei der Zählung der offenen Kapillaren zur Anwendung kamen. Bei Kontrollzählung an unseren Präparaten gemäss den von T. SJÖSTRAND zugrundegelegten Prinzipien resultierten Werte, welche mit denjenigen dieses Forschers völlig übereinstimmen. Die beiden Zählungsprinzipien unterscheiden sich z. B. in Bezug auf Mitrechnung von Kapillaranastomosen, Trennung dichtliegender Kapillaren und Kapillarverzweigungen, Mitrechnung einzelner Blutkörperchen, welche sich nicht mit Sicherheit auf eine quergetroffene Kapillare zurückführen lassen etc.

Da es sich ja nur um relative Werte und keine absoluten Masse handelt, mag man diesen beiden Prinzipien Berechtigung zuerkennen können, unter der Voraussetzung, dass dieselben ähnliche relative Variationen an den Tag bringen. Um dies nachzuprüfen haben wir eine Anzahl von Präparaten mit variierenden Blutmengen nach diesen Prinzipien ausgezählt und dabei gefunden, dass, obwohl die absoluten Werte beträchtlich abwichen, die prozentuale Abweichung zwischen den verschiedenen Präparaten bei beiden Zählungsarten im grossen ganzen dieselbe war. So fanden wir beispielsweise bei Bestimmungen an fünf Tieren aus je zwei Versuchsserien, dass die Werte der einen Reihe bei Zählung nach dem hier angewendeten Prinzip im Mittel um 20.4 % höher lagen als die der anderen, während die entsprechende Differenz bei Zählung nach dem anderen Prinzip 24.5 % war.

Wärmereize.

A. Wärmestrahlung.

Bei Bestrahlung mit der Wärmelampe wurden zwischen 35° und 40° C variierende Hauttemperaturen gemessen. In einigen

Fällen kamen Verbrennungen zustande, wobei die Tiere kassiert wurden. Die Serie enthält nur Tiere ohne makroskopisch nachweisbare Hautveränderungen oder solche mit leichteren Wärmeerythemen.

Der Mittelwert der peripheren Blutmenge beträgt für 11 bestrahlte Tiere 350 000 Blutkörperchen pro mm³ Gewebe in der Leber und 398 000 in der Nierenrinde. Diagramm 1 C macht die Verteilung der Primärwerte ersichtlich.

Die erhaltenen Resultate zeigen, dass lokale Wärmebestrahlung unter diesen Bedingungen nicht zu einer nachweisbaren Zunahme der peripheren Blutmenge in der Leber und Nierenrinde führt. Auch bei denjenigen Tieren, bei welchen Hautschädigungen eingetreten waren, liess sich kein sicherer Effekt feststellen.

Wurden die Versuchsbedingungen in der Weise geändert, dass die Zimmertemperatur von 18° C bei dem obenerwähnten Versuch auf 25—30° C erhöht wurde, dann erhielt man abweichende Resultate. Die Mittelwerte der peripheren Blutmenge in der Leber und Nierenrinde wurden da für 10 Tiere 560 000 bzw. 842 000. Diagramm 1 B zeigt die Anordnung der Primärwerte. Aus diesem Diagramm geht indessen hervor, dass der Mittelwert für die periphere Blutmenge in der Leber auf Grund der grossen Streuung nicht repräsentativ ist.

Der Mittelwert der Bestimmungen der Anzahl offener Kapillaren pro mm² Querschnitt des M. masseter wurde 1320. Die Primärwerte findet man in Tab. 2.

Der mittlere Wert für die Nierenrinde ergibt eine Zunahme mit 130 % des entsprechenden Kontrolltierwerts, während sich eine Veränderung der Kapillarenanzahl im M. masseter nicht sicher feststellen lässt.

Bei diesen Versuchen konnte im Gegensatz zu den Versuchen bei der niedrigeren Zimmertemperatur eine Erhöhung der Rektaltemperatur der Tiere um 1—2° C nachgewiesen werden.

B. Kontaktwärme.

Eine Temperatur des durch den Gummischlauch fliessenden Wassers von ungefähr 50° C erwies sich als der stärkste Wärmereiz, welchen die Haut ohne Verbrennung ertragen konnte. Bei Temperaturen von 55—60° C kamen regelmässig Wärmeschädigungen von derselben Art wie bei Versuchen mit allzu intensiver Wärmestrahlung zustande. Bei Anwendung von 50gradigem

Wasser erhielten wir keine Steigerung der Rektaltemperatur (Zimmertemperatur 18—20° C).

In einer Serie, bei welcher Wasser mit einer Temperatur von 50° C zur Anwendung gelangte, resultierten folgende Mittelwerte (16 Tiere): für die periphere Blutmenge in der Leber 325 000 und in der Nierenrinde 270 000 Blutkörperchen pro mm³ Gewebe. Diagramm 2 D veranschaulicht die Gruppierung der Einzelwerte. Der mittlere Wert für die Anzahl offener Kapillaren im M.masseter (9 Tiere) betrug 1 360 (siehe Tab. 2).

Die erhaltenen Werte weisen keine sicher bestimmbareren Unterschiede gegenüber entsprechenden Werten von Kontrolltieren auf.

Kältereize.

Zwei Tierserien wurden Kältereizen von verschiedener Intensität ausgesetzt. Bei der einen Serie wurde Wasser mit einer Temperatur von +5 — +10° C durch den Gummischlauch geleitet.

Die Mittelwerte der Blutmenge in peripheren Blutgefässen betragen bei dieser Serie (18 Tiere) für die Leber 652 000 und für die Nierenrinde 323 000 Blutkörperchen pro mm³ Gewebe (siehe Diagramm 2 E). Die Anzahl offener Kapillaren pro mm² Masseterquerschnitt ist im Mittel (15 Tiere) 1 710 (siehe Tab. 2).

Diese Werte zeigen eine Zunahme der peripheren Blutmenge in der Leber mit 150 % des entsprechenden Mittelwerts bei den Kontrolltieren, während sich ein sicherer Einfluss auf die Nierenrinde nicht nachweisen lässt. Im M.masseter ist die Anzahl offener Kapillaren im Vergleich zu den Kontrolltieren um 29 % gestiegen. Die Differenz der mittleren Fehler (t) zwischen dieser Serie und der Kontrolltierserie beträgt 8.6, weshalb die Differenz der Mittelwerte statistisch gesichert ist.

Um den Einfluss einer direkten Abkühlung des Leberparenchyms in möglichst grossem Ausmass zu eliminieren wurde der Gummischlauch bei drei Tieren so weit kaudalwärts wie nur möglich angebracht. Hierbei erhielten wir folgende Werte für die periphere Blutmenge: in der Leber 850 000 für sämtliche Tiere und in der Nierenrinde 620 000 bzw. 430 000 und 430 000 Blutkörperchen pro mm³ Gewebe.

Es wurde also auch bei diesen Versuchen ein ähnlicher Einfluss auf die peripheren Blutgefässe der Leber konstatiert. Die periphere Blutmenge in der Nierenrinde weist eine eventuelle Vermehrung auf.

Die Tiere der anderen Serie wurden einem milderen Kältereiz ausgesetzt, indem Wasser mit einer Temperatur von $+15^{\circ}\text{C}$ durch den Gummischlauch geleitet wurde. Hierbei erhielten wir folgende Mittelwerte (9 Tiere): in der Leber 556 000 und in der Nierenrinde 312 000 Blutkörperchen pro mm^3 Gewebe (siehe Diagramm 2 F). In dieser Serie tritt also ein etwas niedrigerer Mittelwert der peripheren Blutmenge in der Leber auf als bei dem stärkeren Kältereiz, aber es wurde gleichwohl eine Verdoppelung gegenüber dem Mittelwert der Kontrolltiere erreicht. Die peripheren Blutgefäße der Nierenrinde lassen auch in diesem Versuch keinen sicheren Einfluss erkennen.

Kältereizung denervierter Haut.

Um ebenso wie bei den früher veröffentlichten Versuchen die Bedeutung der Hautinnervation für die Übertragung des Reizes ausfindig zu machen wurden Experimente über den Effekt von lokaler Kältereizung denervierter Haut angestellt.

Hierbei wurde Wasser mit einer Temperatur von $+5 - +10^{\circ}\text{C}$ verwendet, welches durch den Gummischlauch geleitet wurde. Die Mittelwerte der Bestimmungen der peripheren Blutmenge an 12 Tieren waren: Leber 318 000 und Nierenrinde 271 000 Blutkörperchen pro mm^3 Gewebe (siehe Diagramm 2 G). Die Anzahl offener Kapillaren pro mm^2 Querschnitt des M.masseter betrug im Mittel 1 390 (siehe Tab. 2).

Diese Werte stimmen ja mit den entsprechenden Kontrolltierwerten gut überein. Die Differenz der mittleren Fehler (t) zwischen dieser Serie und der Versuchsreihe mit Kältereizung von Haut mit intakter Innervation beträgt für die Muskelwerte 5.9, weshalb die Differenz der Mittelwerte statistisch sicher ist. *Der Effekt auf periphere Blutgefäße in der Leber und im M.masseter scheint also bei lokalen Kältereizen von einer intakten Hautinnervation abhängig zu sein.*

Einfluss lokaler Hautreize mit Senföl und Ultraviolettstrahlung auf die Anzahl offener Kapillaren pro mm^2 Querschnitt im M. masseter.

A. Intakte Hautinnervation.

Folgende Resultate mögen als Ergänzung der früher veröffentlichten Versuche über den Einfluss lokaler Hautreizung mit Senföl

und Ultraviolettstrahlung auf die periphere Blutverteilung in der Leber und Nierenrinde (G. VON REIS und F. SJÖSTRAND 1938) hier angeführt werden:

Bei lokaler Hautreizung mit Senföl während 20—45 Min. wurde der Mittelwert der Bestimmungen an 12 Tieren 1 500 Kapillaren pro mm² Querschnitt des M. masseter (siehe Tab. 2). Die Differenz der mittleren Fehler bei dieser Serie und der Kontrollierserie beträgt 3,2, weshalb die Differenz der Mittelwerte statistisch gesichert ist. Die Zunahme der Anzahl offener Kapillaren macht im Mittel 13 % aus.

15 Tiere, welche unmittelbar nach 45 Min. dauernder Bestrahlung mit ultraviolettem Licht getötet worden waren, hatten im Mittel 1 520 offene Kapillaren pro mm² Masseterquerschnitt (siehe Tab. 2). Die Zunahme beträgt im Vergleich zu den entsprechenden Kontrolltierwerten hier im Mittel 14 %, und da die Differenz der mittleren Fehler bei diesen beiden Serien 4,4 ausmacht, ist die Vermehrung statistisch sicher.

Reiht man diese Resultate den bei früheren Versuchen über die periphere Blutmenge in der Leber und Nierenrinde bei denselben Zuständen erhaltenen an, so geht hervor, dass die peripheren Blutgefäße sich bei lokaler Hautreizung mit Senföl und Ultraviolettstrahlung sowohl in der Leber, wie in der Nierenrinde und im M.masseter dilatieren.

B. Denervierte Haut.

Bei entsprechender Reizung in Hautbezirken, welche ihrer segmentalen Innervation beraubt worden waren, erhielten wir mit Senföl (8 Tiere) im Mittel 1 260 und bei Bestrahlung mit ultraviolettem Licht (11 Tiere) 1 330 offene Kapillaren pro mm² Masseterquerschnitt (siehe Tab. 2). Die Differenzen der mittleren Fehler bei diesen Serien und den entsprechenden Versuchsreihen von lokaler Hautreizung mit Senföl und Ultraviolettstrahlung an Haut mit intakter segmentaler Innervation betragen 3,4 bzw. 3,2, weshalb die Differenzen zwischen den Serien statistisch gesichert sind.

Diese Werte zeigen, dass man bei Reizung von denervierter Haut keinen sicheren Einfluss auf die Kapillaren im M.masseter erhält, weshalb die obenbeschriebenen Effekte auf diese Blutgefäße durch die Hautnerven vermittelt werden dürften.

Erörterung der Versuchsergebnisse.

Durch die Ausführung der hier beschriebenen Versuche in Narkose wird ausgeschlossen, dass die erzielten Effekte eine Folge von psychischer Exzitation durch die starke Reizung der Sinnesnervenendigungen waren. Als Narkosemittel wurde Pernocton verwendet, welches an sich die periphere Blutverteilung nicht nennenswert verändert (T. SJÖSTRAND 1935, LINDGREN 1935), weshalb wir bei den Versuchen von einem Zustand in den peripheren Blutgefäßen ausgegangen sind, welcher dem bei nichtbetäubten Tieren recht gut entsprechen dürfte.

Der früher vorgenommenen Kontrolle nach (G. VON REIS und F. SJÖSTRAND 1938) bringt die Denervierung keine Zirkulationsstörung in der Haut mit sich, welche beispielsweise für die Entstehung, Freimachung oder Resorption eventueller reizvermittelnder Substanzen Bedeutung haben könnte, weshalb die Versuche demonstrieren dürften, dass der Effekt von lokaler Hautreizung mittels Kälte auf periphere Blutgefäße in der Leber und dem M. masseter des Meerschweinchens durch das Nervensystem vermittelt wird.

Es ist wohl wahrscheinlich, dass der bei lokaler Hautreizung mit Senföl, Ultraviolettstrahlung und Kälte wahrnehmbare Effekt auf die Kapillaren im M.masseter sich nicht auf diesen Muskel beschränkt. Der M. masseter entspricht ja nicht denjenigen Hautsegmenten, in welchen der Hautreiz appliziert worden war, und nimmt offenbar keine Sonderstellung den übrigen Skelettmuskeln gegenüber ein. Es erscheint uns somit berechtigt, die Resultate der Bestimmungen der Anzahl offener Kapillaren in diesem Muskel in gewissem Masse auch auf die übrige Skelettmuskulatur zu übertragen.

Die Übereinstimmung zwischen unseren Resultaten bei lokaler Hautreizung mit Kälte an Meerschweinchen und den von T. SJÖSTRAND an Mäusen bei genereller Kältereizung erhobenen Befunden ist augenfällig. So fand dieser Autor ebenfalls eine Zunahme der peripheren Blutmenge in der Leber, während sich ein sicherer Effekt auf die Nierenrinde nicht konstatieren liess. Diese Übereinstimmung zeigt offenbar, dass man bei Kältereizung in einem kleineren Hautbezirk Reaktionen in den peripheren Blutgefäßen von entsprechender Art wie bei einem generellen Kältereiz auslösen kann. Hinsichtlich Wärme dürften T. SJÖSTRANDS Befunde am ehesten mit den hier beschriebenen Versuchen mit lokaler

Wärmereizung bei höherer Zimmertemperatur zu vergleichen sein, bei welchen eine Erhöhung der Körpertemperatur erzielt wurde. Bei einem solchen Vergleich liegt eine gewisse Übereinstimmung der Resultate vor.

Die bei der vorliegenden Arbeit erhaltenen Resultate stehen allerdings nicht in Einklang zu dem, was man früheren Versuchen mit Plethysmographie nach erwarten sollte. Die Erklärung dieser Verschiedenheit kann teils darin zu suchen sein, dass man bei den erwähnten plethysmographischen Untersuchungen vor allem die Reaktionen der Hautgefäße registriert hatte, welche ja hinsichtlich ihrer speziellen Funktion im Zusammenhang mit der Thermoregulation eine Sonderstellung einnehmen dürften, und teils darin, dass zu- und ableitende Blutgefäße ganz verschieden von den peripheren Blutgefäßen reagieren, und dass die Reaktionen der ersteren Gefäße die der letzteren bei der Plethysmographie verdecken. Letzteres erscheint recht plausibel, da es bei unseren Kältereizungsversuchen auffiel, wie extrem blass die *Organe makroskopisch aussahen, was einer geringen Blutfüllung* der zu- und ableitenden Blutgefäße zugeschrieben werden muss. Dieser Umstand bildet auch ein Beispiel dafür, wie unsicher es sein kann, aus der Farbe eines Organs auf die periphere Blutmenge desselben zu schliessen.

Man kann sich vorstellen, dass eine gesteigerte Durchströmung mit Blut durch zu- oder ableitende Blutgefäße durch eine raschere Zirkulation in den peripheren Blutgefäßen ohne eine Zunahme der peripheren Blutmenge zustandekäme, und zwar infolge von Reaktionen in zu- und ableitenden Blutgefäßen und daraus folgenden veränderten Druckverhältnissen. So kann man sich eine vermehrte Durchströmung lediglich als Folge einer Dilatation von Arterioli denken. Deshalb lassen sich bei der Registrierung mittels Thermostromuhr keine sicheren Schlussfolgerungen über die periphere Blutverteilung ziehen.

Ein Gegenstück in den peripheren Blutgefäßen zu der Steigerung der Durchströmung in der Vena renalis, welche REIN, REIN und RÖSSLER bei genereller Abkühlung konstatiert haben, konnten wir bei lokaler Kältereizung und auch T. SJÖSTRAND bei genereller Abkühlung nicht nachweisen, was, von dem oben angeführten abgesehen, darauf zurückzuführen sein könnte, dass man sich einen vermehrten Blutstrom durch die Nieren z. B. durch die Glomeruli vorstellen kann, ohne eine entsprechende Dilatation der peripheren Blutgefäße rings um die Tubuli.

Der von REIN, REIN und RÖSSLER konstatierten erhöhten Durchblutung durch die Vena porta scheint dagegen eine Zunahme der peripheren Blutmenge in der Leber zu entsprechen, welche man sowohl bei genereller (T. SJÖSTRAND 1935), wie den vorliegenden Versuchen nach bei lokaler Kältereizung erhält.

Es ist auffallend, dass bei Hautreizen verschiedener Art die peripheren Blutgefäße in den hier studierten Organen in hohem Grade verschieden beeinflusst werden. So zieht Hautreizung mit Senföl und Ultraviolettstrahlung eine Zunahme der peripheren Blutmenge in sowohl der Leber, wie in der Nierenrinde und Muskulatur nach sich, während ein Kältereiz auf dieselben Blutgefäße nur in der Leber und Muskulatur einwirkt. Auch starke Wärmereize geben keinen nachweisbaren Effekt, ausser im Zusammenhang mit Hyperthermie, und dann hauptsächlich in der Nierenrinde. Im letzten Fall ist nicht untersucht worden, ob der Effekt von intakter Hautinnervation abhängt. Diese Verhältnisse scheinen zu der Annahme zu berechtigen, dass die Wirkungen von Hautreizen mit verschiedenen Reizmitteln in verschiedener Weise innerhalb des Nervensystems vermittelt werden können.

Zusammenfassung.

Die periphere Blutmenge in der Leber und Nierenrinde sowie die Anzahl offener Kapillaren pro mm² Querschnitt des M. masseter wurde an Meerschweinchen nach lokaler thermischer Hautreizung in Pernoctonnarkose bestimmt. Früher veröffentlichte Bestimmungen der peripheren Blutverteilung in der Leber und Nierenrinde nach Einwirkung von lokaler Hautreizung mit Senföl und Ultraviolettstrahlung wurden durch Bestimmungen der Anzahl offener Kapillaren pro mm² Masseterquerschnitt ergänzt.

1. Lokale Bestrahlung mit einer Wärmelampe brachte keinen nachweisbaren Effekt auf die periphere Blutverteilung in der Leber und Nierenrinde mit sich, ausser im Zusammenhang mit dem Eintreten von Hyperthermie (1—2° C), wo sich eine Steigerung der peripheren Blutmenge in der Nierenrinde mit 130 % der entsprechenden Kontrolltierwerte nachweisen liess. Ausserdem lag ein infolge von Streuung der Werte nicht sicherer Einfluss auf die Leber vor, während kein Effekt auf die peripheren Blutgefäße im M. masseter festgestellt werden konnte.

2. Lokale Wärmereizung mittels Kontaktwärme (ohne Steige-

rung der Rektaltemperatur) hatte keine sichere Beeinflussung der peripheren Blutgefäße in der Leber und Nierenrinde zur Folge.

3. Bei lokalen Kältereizen stieg die periphere Blutmenge in der Leber mit 150 % des entsprechenden Kontrolltierwerts, während sich ein Einfluss auf die Nierenrinde nicht beobachten liess. Die Anzahl offener Kapillaren im M. masseter nahm um 29 % zu.

4. Lokale Kältereizung von denervierter Haut brachte keinen sicheren Einfluss auf periphere Blutgefäße in der Leber, Nierenrinde und im M. masseter mit sich, woraus gefolgert wird, dass der Effekt auf die Leber und Muskulatur bei Kältereizung der Haut durch die Hautnerven vermittelt wird.

5. Die Anzahl offener Kapillaren im M. masseter stieg bei:

a) lokaler Hautreizung mit Senföl um 13 %, und

b) lokaler Bestrahlung mit ultravioletttem Licht um 14 %.

6. Diese Effekte blieben aus, wenn die Hautreizung mit Senföl oder Ultraviolettstrahlung auf Haut appliziert wurde, welche ihrer segmentalen Innervation beraubt worden war.

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Tension Changes during Tetanus in Mammalian and Avian Muscle.¹

By

U. S. von EULER and ROY L. SWANK.²

(With 13 figures in the text.)

The tension developed by a mammalian voluntary muscle during a tetanus elicited by indirect stimulation of a moderate frequency usually becomes maximum quickly, and after a "plateau" of varying duration gradually diminishes. In a rested white muscle this tension may remain approximately maximum for some 10 seconds, but the tension developed by a second tetanus which follows the first after a short interval rapidly declines because of fatigue, although it may yield the same or even higher initial tension.

Under similar experimental conditions with the tibialis muscle stimulated indirectly by a maximal tetanizing current at a frequency of about 45 p.s., a somewhat different tension curve may be observed. This is characterized by the usual quick rise followed by a momentary decrease in tension and then a secondary rise, which may attain a considerable height.

Some of the features of this phenomenon and the conditions governing its appearance have been investigated and will be described in the present paper.

Methods.

Cats and pigeons have been used as experimental animals. In the majority of cases the animals were decerebrated while

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anesthetized with ether. The tibialis anticus (white muscle) or soleus (red muscle) of the cat, were prepared and its tendon connected to a spring myograph. The leg was firmly fixed in a BROWN-SCHUSTER myograph stand. The muscle tension was recorded on a smoked drum or on photographic paper.

The muscle was stimulated indirectly through shielded silver electrodes on the sciatic nerve (see diagram, fig. 1) by means of neon lamp stimulators, acting on two pairs of electrodes, one pair for single shocks, and the other (central) pair for tetanic stimulation. All stimuli were just supramaximal. The stimulating cathode was placed nearest the muscle.

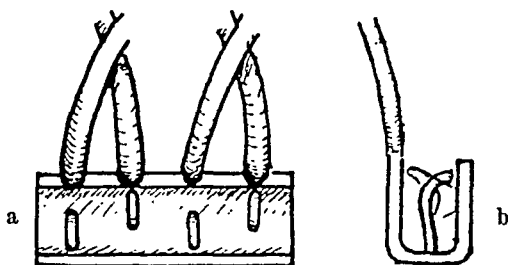


Fig. 1. Stimulating electrodes, a from above, b from the side. The nerve is slipped on the silver electrodes, emerging from the floor of the ebony shield.

Precautions were taken to keep the animal warm and the muscle warm and moist.

In the experiments on denervated muscles the sciatic nerve was cut 5 to 16 days previously and the muscle stimulated directly by silver pin electrodes inserted about 3 cm. apart in the belly of the muscle.

Action potentials were led off from the belly and tendon of the muscle by silver pins and recorded by means of an ABRAMSON torsion band oscillograph (WEBER, 1939) fed from a condenser coupled amplifier of the type described by MATTHEWS (1938). The oscillograph and myograph light was derived from a 4 volt lamp and accumulator and the whole recording system was contained in a case 50 × 30 × 30 cm. (see diagram).

In a few cases action potentials were recorded with a cathode ray oscillograph.

Close arterial injections were made according to the technique described by BROWN (1938).

Time was recorded in $\frac{1}{50}$ th second with a stronger mark at every $\frac{1}{10}$ th second.

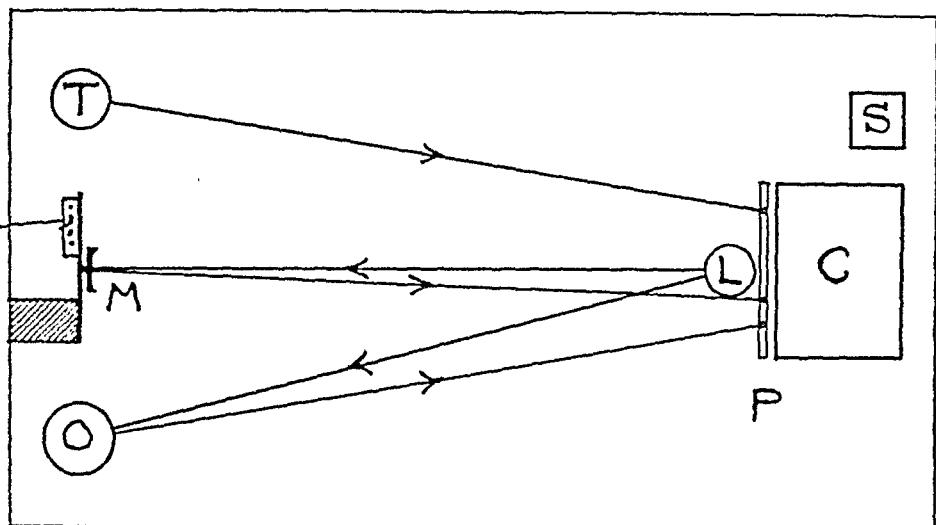


Fig. 2. Diagram of recording apparatus (Euler-Petersen). C camera, L 2 W lamp, M Myograph, O Oscillograph, P projection screen for inspection, T time marker.

Experimental Results.

I. Cats.

A. Tibialis Anticus Muscle.

The appearance of the secondary rise in tetanus tension.

The effect to be described we have termed, for the sake of brevity "secondary rise".

A typical secondary rise in tetanus tension in the tibialis anticus muscle is illustrated in fig. 3. The character of the rise varied in details, especially concerning its height, which was sometimes hardly perceptible, and at other times showed an increase in tension of 0.8 kg. (Fig. 5 a.) Variations also occurred with regard to the initial dip, which was well-marked, small or absent. The appearance of the »dip» was to some extent accentuated by the inevitable over-throw due to the inertia of the lever in the mechanical recording system, but this did not account for the whole effect.

The increase in tetanus tension usually appeared after a few seconds of stimulation and lasted for some 10 sec., but had in some cases a longer duration, although it seldom exceeded 15 sec.

The most typical examples of secondary rise occurred, as a rule, one hour or longer after the animal had been prepared. We also had the impression that the effect was more pronounced if the

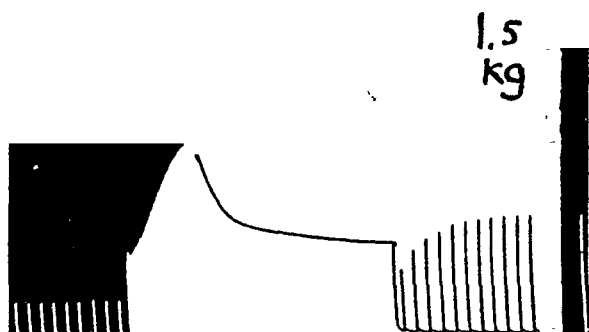


Fig. 3. Cat, chloralose anesthesia, tibialis anticus. Indirect maximal single shocks, 3 sec. apart. Maximal tetanus at 30 per sec. for 1 min:

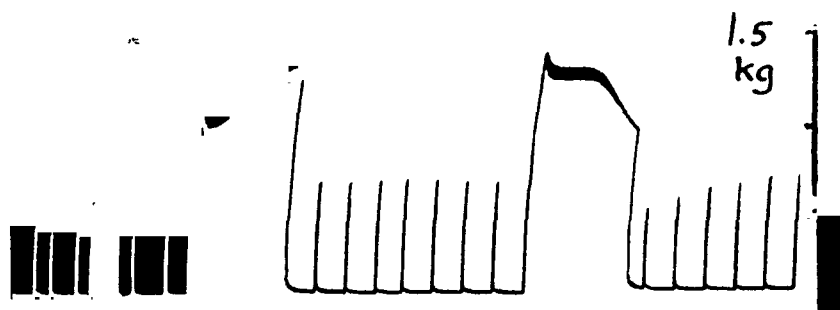


Fig. 4. Cat, decerebrated, tibialis anticus. Maximal motor nerve single shocks 2.9 sec. apart. Two 10 sec. tetani at 45 per sec. interval 1 min. First tetanus 9 min after the preceding one.

single (maximal) twitch tension was low, as it usually was in preparations used for several hours.

Influence of stimulating frequency and recovery period.

It soon became evident that the phenomenon of the secondary rise could be most regularly demonstrated when a stimulating frequency of about 40—50 p.s. was used. At both lower and higher frequencies the effect was less conspicuous, and with frequencies below 25 p.s. and above 70 p.s. it was usually absent.

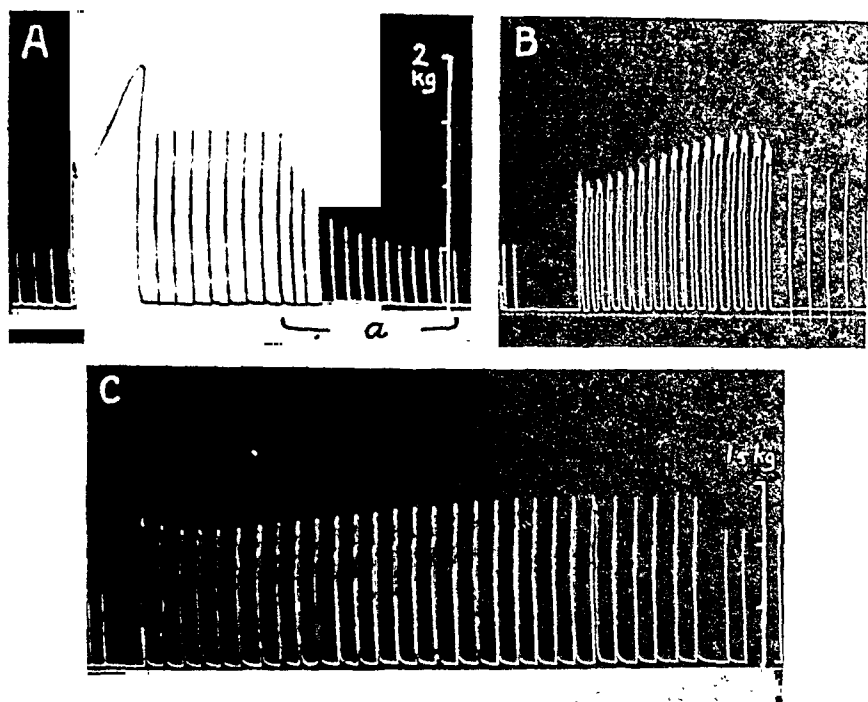


Fig. 5. Cat, decerebrated, tibialis anticus. Maximal motor nerve single twitches 2.8 sec. apart. A. motor nerve tetanus 45 per sec. for 10 sec. Interval between shocks during (a) 1 min. B. series of 18 tetani at 45 per sec. 0.6 duration, 1 sec. interval. C. tetani at 45 per sec., 0.4 sec. duration, 3 sec. interval. Before and after the tetani single shocks.

It was also found that the interval between tetani was of great importance. If the time elapsing between two tetani was less than one minute the secondary rise was greatly diminished or absent during the second tetanus. As this interval was increased the secondary rise became more pronounced and reached a maximum when the interval was 7 to 10 minutes or longer. The failure of a second tetanus after a very short interval to show the effect may be explained by the fact that the initial tension of the second tetanus was already maximum or nearly so (fig. 4).

Action of interrupted tetani.

The development of the secondary increase in tension was clearly demonstrated by using intermittent short tetani. Fig. 5 B shows the gradual increase in tension which appeared when the muscle was stimulated indirectly 18 times at 2 sec. intervals with tetani of $\frac{1}{2}$ —1 sec. duration. The second short tetanus shows a

decrease in tension as compared with the first one, but a definite and gradual increase in tension to maximum takes place in the following tetani which occupy a period of 25 seconds. Following this increase the tension falls in subsequent tetani.

If the intervals between tetani of 1 second duration is increased to 10 seconds the secondary rise is barely detectable, but at 3 sec. intervals the initial decrease as well as the following increase in tension is well established even when tetani of $\frac{1}{2}$ sec. are used (fig. 5 c). Shorter intervals between tetani do not appreciably alter the form of the usual secondary rise curve (fig. 6).

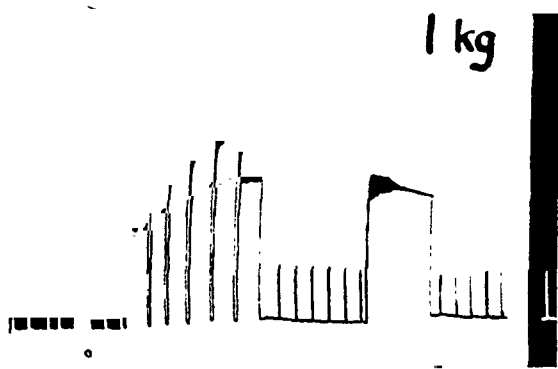


Fig. 6. Cat, decerebrated, tibialis anticus. Sciatic nerve cut 6 days previously. Direct stimulation 50 per sec. First tetanus interrupted for 0.5 sec. every 3 sec. Second tetanus 20 sec. after the first one.

Effect on denervated muscle.

In order to determine if the phenomenon was dependent for its appearance upon an intact neuromuscular connection, we have directly stimulated muscles previously denervated. In four such experiments the secondary rise phenomenon was clearly present (fig. 6).

Relation between the secondary rise and the single twitch tension after a tetanus.

It was regularly observed that the appearance of the secondary rise was closely related to the post-tetanic potentiation as described

by BROWN and EULER 1938, and others. Thus a post-tetanic potentiation was always observed in those cases where a secondary rise could be produced whereas a secondary rise in muscle tension was regularly absent when no post-tetanic potentiation appeared as a result of a preceding tetanus. The functional relationship between these two phenomena is further stressed by the fact that a secondary rise could not be produced effectively in a second tetanus unless the post-tetanic potentiation had disappeared or greatly decreased. Usually the p. t. p. was present for 5—10 minutes after a 10 second tetanus. The muscle tension elicited by single slightly supramaximal stimuli at regular intervals therefore served as an accurate indication of the degree of recovery of a muscle from tetanus (fig. 5 A).

Sometimes the first few single twitches which followed a tetanus with a secondary rise were smaller than the ones which followed (fig. 2), thus causing a delay in the development of the post-tetanic potentiation. On the other hand this delay in the appearance of the p.t.p. has been observed to some degree during a second tetanus when a secondary rise was absent, and it has also been observed after potassium injections, and after curare.

Action of potassium injections.

The relationship of the secondary rise phenomenon and the post-tetanic potentiation led us to test the influence of potassium upon it. Potassium chloride was administered intravenously in isotonic solution and by way of close arterial injections. In our experiments this procedure decreased or abolished the secondary rise. The doses used were 1 to 5 mg. KCl, which produced a twitch and a subsequent increase in the single muscle twitch tension for some time, as described by BROWN (1937). The effect of single small doses of KCl intraarterially upon the initial tetanus tension was negligible, whereas repeated doses or big single doses produced a lasting decrease in tension.

Action of curare.

In several experiments the secondary rise phenomenon was studied in muscles that were gradually being curarized. When the curare action had developed to such a degree that the single twitch tension was definitely reduced the secondary rise during the tetanus was also reduced. Although the initial tetanus tension

was fairly well maintained there was only a small secondary increase in tension or none at all during the tetanus and a second tetanus, at a later stage of the curare action, showed no sign of a secondary increase in tension (cf. FENG et al. 1933).

B. Experiments on the Soleus Muscle.

Since the tibialis anticus muscle of the cat belongs to the group of "white" muscles, which develop a quick contraction, we thought it desirable to investigate the secondary rise phenomenon on a "red" muscle. For this purpose the soleus muscle was used.

The behaviour of this muscle in a tetanus differs in several respects from that of tibialis anticus. Thus the tetanic tension is usually high compared with the single twitch tension; a smooth tetanus is obtained with a tetanizing frequency of less than 15 p.s.; the tension is sustained for a much longer time; and the same type of tension curve may be repeated after a short interval.

The same frequency of stimulation which produced a secondary rise in the tibialis was ineffective in this respect on the soleus. When the stimulating frequency was lowered to 15—25 per second a slight rise in tension occurred during the tetanus, but it was much less marked than that observed in the tibialis (fig. 7). In fact we could never be certain that the response of the two muscles was of the same type. Contrary to observations on the tibialis two or more tetani in rapid succession produced almost identical responses in soleus, thus giving us another indication that the action of these muscles is different. In the tibialis the secondary

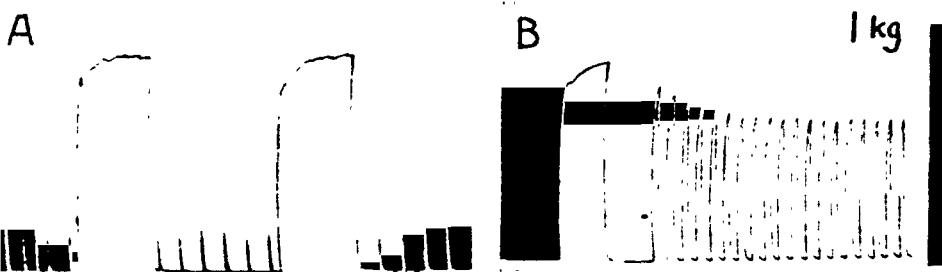


Fig. 7. Cat, decerebrated, soleus. A. Two maximal motor nerve tetani at 18 per sec. for 10 sec. 17 sec. apart, maximal single shocks 3 sec. apart. B. Tetanus 15 per sec. followed by series of tetani of 1 sec. duration and 1 sec. apart.

rise was accompanied by a post-tetanic potentiation of the subsequent single twitches, and a series of short tetani in rapid succession showed an increase in tension. Since neither of these actions occurred as a result of low frequency stimulation of the soleus we are inclined to believe that the small tension rise in this muscle under the conditions mentioned is different in character from that in tibialis.

C. Action Potentials.

Muscular action potentials have also been recorded both alone, and simultaneously with the mechanical records during the development of the secondary rise. Because of the finding of BROWN (1937) and BROWN and EULER (1938) that an increase in muscle tension after an injection of potassium or following a short tetanus may be associated with a decrease in action potential this seemed especially desirable. The similarity of the behaviour of the action potentials in these two instances was one of the reasons for assuming that they were, in principle, due to a similar kind of change in the muscle, e. g., a shift in the potassium ions of the muscle. In the present experiments we have found a more or less pronounced decrease in spike height during the increased tension constituting the secondary rise, fig. 8 illustrates this point. We have also confirmed the observation of BROWN and EULER that the increased muscle tension in a maximal single twitch following a tetanus is regularly associated with a decrease in the action potentials of the whole muscle.

During a tetanus the action potentials usually decreased gradually as the muscle tension increased, but in a few cases in which the secondary rise was weak or absent the muscle action potentials failed to decrease. As a rule, however, the action potentials fell slowly at first and sharply during the final fall in muscular tension which followed the secondary rise. We have never observed an increase in a p. during the secondary rise if the stimuli were maximum.

During a series of short tetani there is a gradual increase in the maximum tension developed by each tetanus so that a secondary rise in tension results. During this increase in tension the action potentials gradually diminish, and later when the muscle tension starts to fall the action potentials decrease even more rapidly. Finally when the muscle tension has fallen back to the initial value (e in fig. 9) the a. p. may be decreased by some 30 %. In (f) the

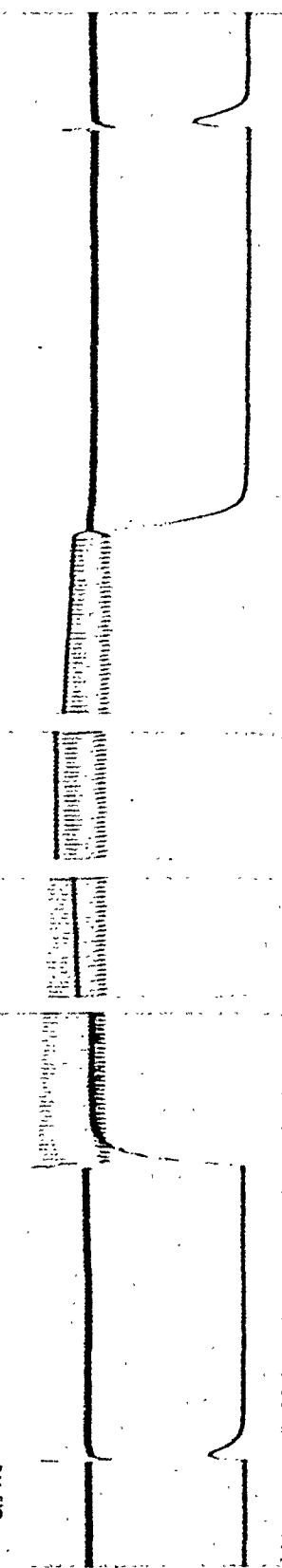


Fig. 8. Cat, decerebrated, tibialis anticus. Upper curve action potentials, belly-tendon lead. Lower curve myogram. Maximal indirect tetanus at 45 per sec. for 12 sec. Maximal motor curve single shocks before and after the tetanus. (The beginning, 4 and 9 sec. after, and the end of the recording are shown in the figure.)

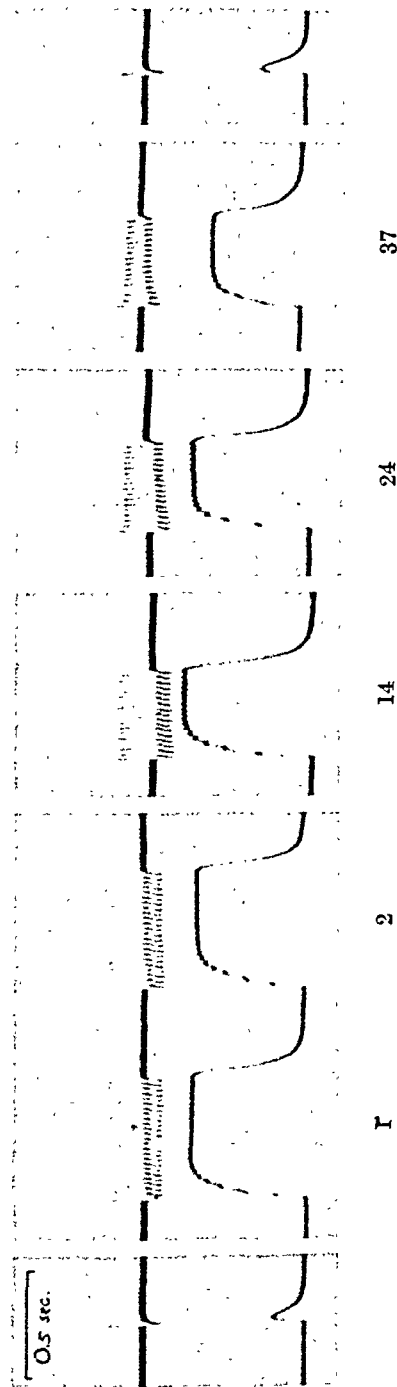


Fig. 9. Cat, decerebrated, tibialis anticus. Upper curve action potentials, belly-tendon lead. Lower curve myogram. 37 maximal indirect tetani at 44 per sec. for about 0.4 sec. each with 0.4 sec. intervals. Tetani nr 1, 2, 14, 24 and 37 are shown. Single maximal motor nerve shocks before and 1 sec. after the series of tetani.

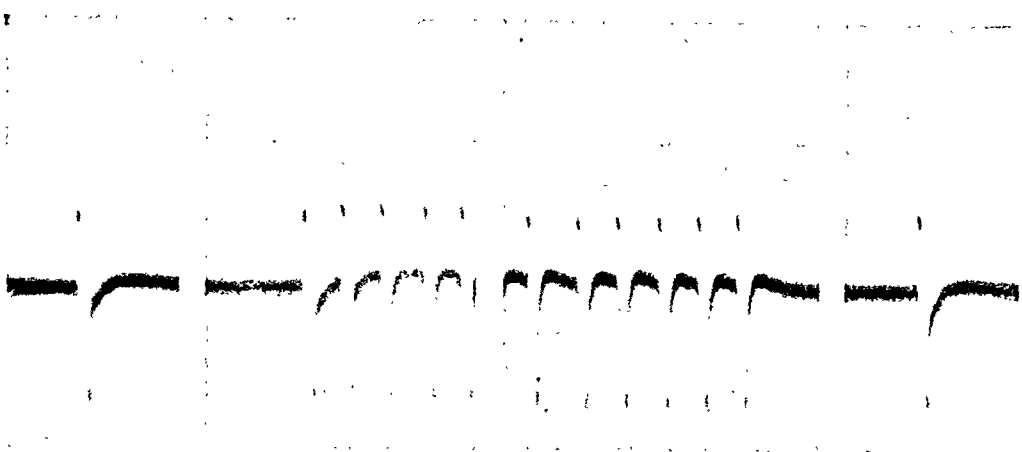


Fig. 10. Cat, decerebrated, soleus. Action potentials before and after, and at the beginning and end of a 5.5 sec. tetanus at 17 per sec. Time $1/5$ sec. (cathode-ray oscillograph).

muscle tension and a. p. have fallen even more, and the single muscular twitch about 3 secs. after the last tetanus was characteristically potentiated and the a. p. reduced. From the curves it is obvious that the first contraction or the first and second ones in each tetanus bears a close relation to the maximum tetanic tension (except in the last tetanus, f.) but do not correspond to the first action potential. However, the first contraction of the last tetanus and the following single twitch are in close agreement with regard to muscle tension and action potential. It seems that the alterations in the muscle which lead to the subsequent appearance of a post-tetanic potentiation develop in a way which can be followed by observing the separate action potentials, and the first contraction of each tetanus. The curves b and c in fig. 9 also make clear that the frequently occurring initial fall in muscular tension during a series of tetani is due to a decrease in the first part of the second tetanus curve. As soon as the first contraction of the tetanus is increased the maximal tension also increases and the a. p. diminishes. The time necessary for the secondary rise to appear is approximately the same as that necessary for the post-tetanic potentiation to develop, i. e. 1—2 sec.

In the soleus, however, we have not observed any definite changes in the action potential during tetani of 5—10 sec. duration at frequencies 15—25 per sec. though there was the usual rise in tension. The action potential accompanying the first single twitch about 1 sec. after the tetanus was mostly unchanged, (fig. 10),

and so was the tension of the twitch. We take this as an indication that the gradual rise of tension in the soleus during a low frequency tetanus is of another kind than in the tibialis.

II. Tension Changes during Tetanus in the Tibialis Anticus Muscle of the Pigeon.

The secondary rise in tension which developed during tetanus in the tibialis anticus muscle of the cat was also observed in the corresponding muscle of the pigeon. The response occurred regularly in this muscle of decerebrated pigeons when tetanizing frequencies of the same order as those used in the experiments on the cat's tibialis muscle were used (fig. 11 and 12). Certain differences have been noted, however. Whereas the second of two tetani in quick succession does not produce a secondary rise in the tibialis

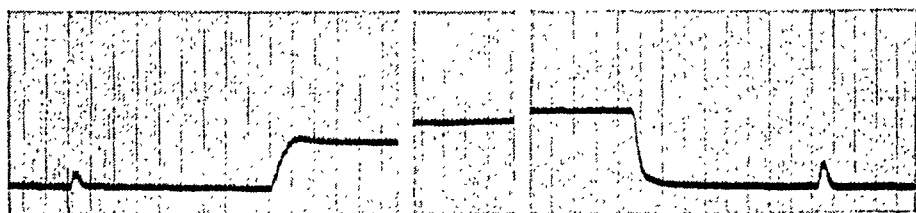


Fig. 11. Pigeon, decerebrated, tibialis anticus. Myogram of a sec. 6 indirect maximal tetanus at 45 per sec. Time 0.1 sec.

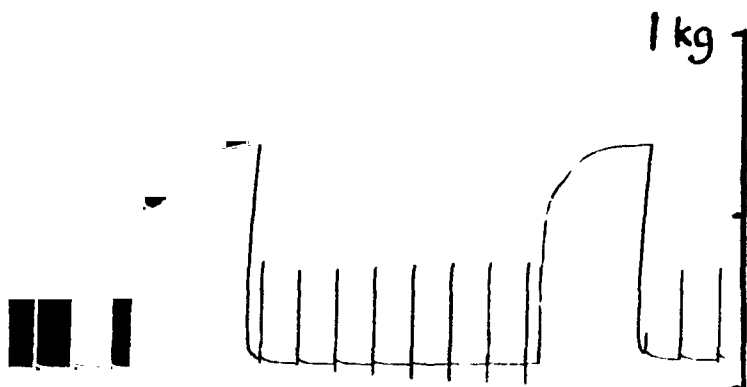


Fig. 12. Pigeon, decerebrated, tibialis anticus. Two 10 sec. maximal indirect tetani 20 sec. apart at 45 per sec. Single shocks 3 sec. apart. 8 min rest before first tetanus.

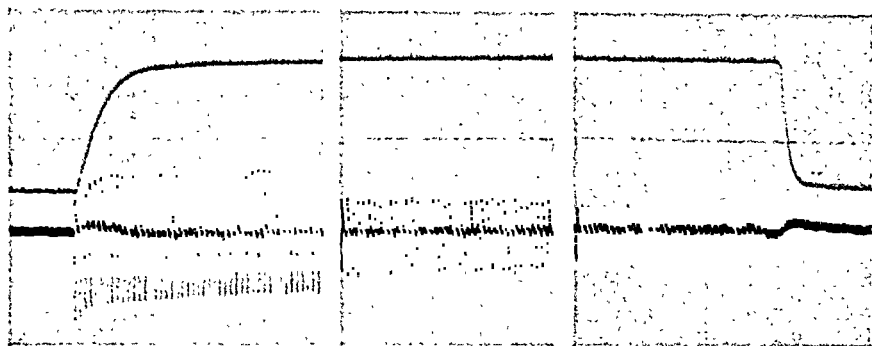


Fig. 13. Pigeon, decerebrated, tibialis anticus. Upper curve myogram, lower curve action potentials. Tetanus 10'' at 40 per sec. Time 0.1 sec. The first, middle and last part of the curve is shown.

of the cat and is also less effective with regard to the production of a post-tetanic potentiation, the second tetanus in the pigeon showed a definite secondary rise (fig. 12) under the same conditions. However, the second tetanus was frequently followed by a slight depression of the subsequent single twitches.

The action potentials during tetanus sometimes showed a striking decrease in spike height during the rise in tension (fig. 13). There was also a definite reduction in the size of the a. p. of the single twitch following the tetanus in spite of an increase in tension. Both of these observations are in full conformity with the results on mammalian muscle. During a second tetanus the decrease in voltage of the action potentials occurred more rapidly than during the first tetanus.

BROWN and HARVEY (1938) have reported that the second of two muscular action potentials separated by a short interval in the gastrocnemius muscle of the hen anesthetized with pernocton, was larger than the first. This was observed occasionally, but as a rule there was no difference in the spike height between the first and the second action potential in a tetanus yielding discrete action potentials (40—45 p. sec.), (fig. 13).

Discussion.

The secondary rise phenomenon described in this paper obviously bears definite relations to the potentiating effect of a tetanus on subsequent single muscle twitches as reported by GUTTMAN et al. (1937), ROSENBLUETH and MORISON (1937), FENG et al. (1938),

BROWN and EULER (1938) and PIRQUET (1938). These relations may be summarised in the following points.

(1) The secondary rise in tension in a tetanus, elicited by a suitable indirect or direct stimulation of the tibialis of the cat or the pigeon is regularly followed by a potentiation of subsequent single twitches.

(2) In cases where the post-tetanic potentiation is absent no secondary rise occurs (injection of KCl in sufficiently large doses; in a second tetanus within a short time after the foregoing; and in soleus at low tetanizing frequencies).

(3) The action potential of a single twitch following a tetanus is usually depressed though the twitch tension may be increased up to 100 p.c., and the action potential during a secondary rise show a gradual decrease in spike height. We have observed a decrease of more than 50 % of the spike height of the action potential at a time when tension of the muscle was distinctly raised. It will appear that the altered state in the muscle, which produces a post-tetanic potentiation functions during tetanus to produce an increase in muscular tension.

In addition to other factors which influenced the development of the secondary rise, i. e. frequency of stimulation, and interval between tetani, the condition of the preparation seems to be important. We have not been able to find a definite relationship between the initial tetanic tension and the secondary rise, although, admittedly, the phenomenon was usually more striking in cases where the initial tetanic tension was not too high. This was not due to a submaximal stimuli, for an increase in stimulus strength did not change the configuration of the curve. The initial tetanic tension at moderate frequencies generally decreases somewhat in our experiments an hour or so after the preparation of the muscle and it was then that the secondary rise phenomenon was most easily demonstrated. This "aging" process also occurred during a period of rest, with the muscle unhooked and kept warm, and under these circumstances the effect was often very conspicuous. We feel that this change cannot be explained solely on a circulatory basis.

There are obviously two possible explanations for the slowly developing rise in tension during a tetanus, namely, (1) a greater number of fibres entering into action simultaneously and (2) an increase in the tension of each fibre. Of course, these events may appear together. In a few experiments a slight initial increase

occurred in the size of the action potential during tetanus indicating, perhaps, that more fibers were becoming active. The action of the tetanus here is clearly the same as is seen in a partially curarized muscle. On the other hand in most experiments the action potentials decreased from the beginning of the tetanus. It would appear difficult to decide if the size of the action potentials gives any definite information as to the number of fibers partaking in the contraction.

A decrease in the amplitude of the action potentials during a tetanus could be due, at least partly, to temporal dispersion. We have observed a marked reduction in spike height, however, without any noticeable change in the width of the action potential, and have also observed a definite broadening of the potential with only a small reduction in size. It should also be recalled that there is often no sign of an increased temporal dispersion in the smaller action potential accompanying the potentiated twitch which follows a tetanus.

From the beginning of a tetanus the time necessary for a secondary rise in tension to appear is usually about 2 seconds. During the first second, there is frequently a slight fall in tension, without significant changes in the action potentials. The reason for this dip is not clear, but we may regard it as the interference minimum of two curves, one falling (fatigue) and one rising (specific effect). At any rate we have no evidence for a temporary reduction in the number of active fibers, since the action potential is, as a rule, unaltered during this stage. If we compare the first three contractions of the first, second, and third tetani in fig. 9, we find that this combined tension is lowest in the second tetanus. In the third tetanus (not shown in the figure) the first contraction is already increased, indicating a change in the state of the muscle though the total tension is still slightly less than was attained by the first three contractions of the first tetanus. Subsequent to the third tetanus the first contraction in each tetanus remains slightly elevated, although there is a slight final regression, probably due to fatigue. It therefore appears that after the two first tetani a change has taken place in each muscle fiber so that it contracts with greater force. The total tetanic contraction is also dependent upon other factors such as the amount of available contraction material, etc.

In the muscle of the pigeon the period of depression or fatigue is less obvious and the muscle is able to produce a secondary

rise after a very short interval. No doubt this indicates a quicker restoration to the pretetanic conditions, and it correlates with the fact that the post-tetanic potentiation lasts for a much shorter time in the pigeon than in the cat.

In the soleus we have not noted any changes in the action potentials during the rise in tetanic tension, using low stimulating frequencies and it thus seems that the mechanism for the rise is different in this muscle. We have confirmed the observation of BROWN and EULER that the post-tetanic potentiation was less easily elicited in this muscle. Thus an interpolated tetanus of 2—4 seconds duration at a moderate frequency did not, as in the tibialis, lead to an increased tension of the following twitch but, reversely, to a decrease. This is in keeping with our observations that a series of short tetani of the soleus result in a gradual fall in tension of this muscle, whereas a complete recovery is obtained in some 5 or 10 seconds when suitable tetanizing frequencies are used. When much higher frequencies were used even a short tetanus on soleus often produced a post-tetanic potentiation, as shown by FENG et al. (1938). On these occasions there was also a clear secondary rise, which strengthens our view that these two phenomena are causally related.

On similar grounds as those presented by BROWN and EULER for the post-tetanic potentiation we cannot consider the secondary rise similar to the Orbeli phenomenon.

The expenses of this investigation have been defrayed by a grant from the Therese and Johan Andersson Memorial Foundation to one of us (U. S. v. E.).

Summary.

Indirect maximal tetanic stimulation of the tibialis muscle of the cat and the pigeon at frequencies of about 45 p. sec. frequently lead to a gradual increase in tension of the muscle to a considerable extent.

This secondary rise in tension is accompanied by a diminution of the action potentials. When a series of short tetanic stimulations are applied to the nerve the maximal tension of each tetanus shows, after a short depression, a gradual increase up to a maximum followed by a fall.

In the soleus muscle of the cat this phenomenon is much less obvious at frequencies of 15—40 p.s. and there are only slight changes in action potentials at these stimulating frequencies.

The phenomenon described here appears to be closely related to the post-tetanic potentiation of a single muscle twitch.

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Pyrophosphatase im Blut.¹

Von

KNUT SJÖBERG.

Das Enzym, Pyrophosphatase, welches anorganische Pyrophosphate und organisch gebundenes Pyrophosphat in anorganische Orthophosphate überführt, wurde von KAY in verschiedenen Organen und in kleiner Menge im Blut nachgewiesen. Auch ROCHE konstatierte das Vorkommen eines derartigen Enzyms in den Blutkörpern und im Serum. Irgendwelche Angaben über die Variationen der Enzymaktivität bei verschiedenen Tierarten und unter verschiedenen Umständen bei Tieren wurden jedoch nicht gemacht.

Das Vorkommen verschiedener Stoffe im Blut kann teils darauf beruhen, dass diese daselbst eine gewisse Funktion zu erfüllen haben, teils dass sie intermediäre Stoffwechselprodukte bilden, die mit Hilfe des Blutes zwischen den verschiedenen Geweben und Organen transportiert werden. Letzteres gilt besonders für gewisse im Serum vorkommende Verbindungen. Da sich die Pyrophosphatase, wie später gezeigt werden wird, hauptsächlich in den Erythrozyten vorfindet, scheint dieselbe bei den Stoffwechselprozessen in diesen eine gewisse Aufgabe zu haben. LOHMANN hat nachgewiesen, dass das Blut und besonders die Erythrozyten organische Pyrophosphatester in Form von Adenylpyrophosphat enthalten, das als Substrat für die Pyrophosphatase dienen kann. Sicher kommen auch andere organische Pyrophosphorsäureester vor. So wird ja beispielsweise das Aneurin an Pyrophosphat gekoppelt und bildet Cocarboxylase.

Mit Rücksicht auf die grosse Bedeutung, die derartigen Pyrophosphatestern und deren Synthese und Hydrolyse zukommt,

¹ Der Redaktion am 6. August 1940 zugegangen.

wird hier über eine Methode zur Bestimmung von Pyrophosphatase im Blut und Blutserum bzw. Plasma nebst einer vorläufigen Untersuchung über das Vorkommen dieses Enzyms im Blut des Menschen und verschiedener Tierarten berichtet. Im Zusammenhang hiermit wurde die Abhängigkeit der Pyrophosphatase von der Wasserstoffionenkonzentration und ihre Veränderungen bei der Aufbewahrung der Blutproben *in vitro* untersucht. Schliesslich wurden die Pyrophosphat- und Pyrophosphatasemengen im Blut miteinander verglichen.

Versuchsmethodik.

Als Substrat wurde Natriumpyrophosphat angewandt, und die Enzymwirkung wurde durch Bestimmung des während einer gewissen Zeit freigewordenen Orthophosphats nach FISKE und SUBBAROW festgestellt. Da grössere Mengen Natriumpyrophosphat die Bildung der blauen Verbindung hemmten, die hierbei aus Ammoniumphosphormolybdat und dem Reduktionsmittel entsteht, musste die Menge des Pyrophosphats in der Reaktionsmischung relativ niedrig gehalten werden.

Folgende Reaktionsmischung erwies sich als geeignet:

$\text{Na}_4\text{P}_2\text{O}_7$ -Lösung	4 ml
MgSO_4 -Lösung	4 ml
Boratpuffer	10 ml
Blut bzw. Serum oder Plasma . . .	4 ml
Aq. dest.	18 ml.

Zusammensetzung der Lösungen:

$\text{Na}_4\text{P}_2\text{O}_7$ -Lösung. 4.000 g $\text{Na}_4\text{P}_2\text{O}_7$ + 10 Aq. für analytische Zwecke werden in 900 ml dest. Wasser gelöst. Zusatz von so viel HCl, dass $\text{pH} = 7.4$ wird, danach Verdünnung auf 1 000 ml mit dest. Wasser. Die Lösung zerteilt sich allmählich bei der Aufbewahrung. Überschiebt man die Lösung mit Petroleumäther und bewahrt sie bei niedriger Temperatur auf, so kann sie sich einige Wochen lang brauchbar halten.

MgSO_4 -Lösung. 5.00 g reines MgSO_4 + 7 Aq. werden in 1 Lit. dest. Wasser gelöst.

Boratpuffer nach Palitzsch $\text{pH} = 7.4$. 10 Teile M/20 Borax + + 90 Teile M/5 Borsäure, M/20 NaCl.

Die Bestimmung wird bei $37\text{--}38^\circ \text{C}$. im Wasserthermostaten ausgeführt. Nach gewissen Zeitintervallen, zweckmässig nach

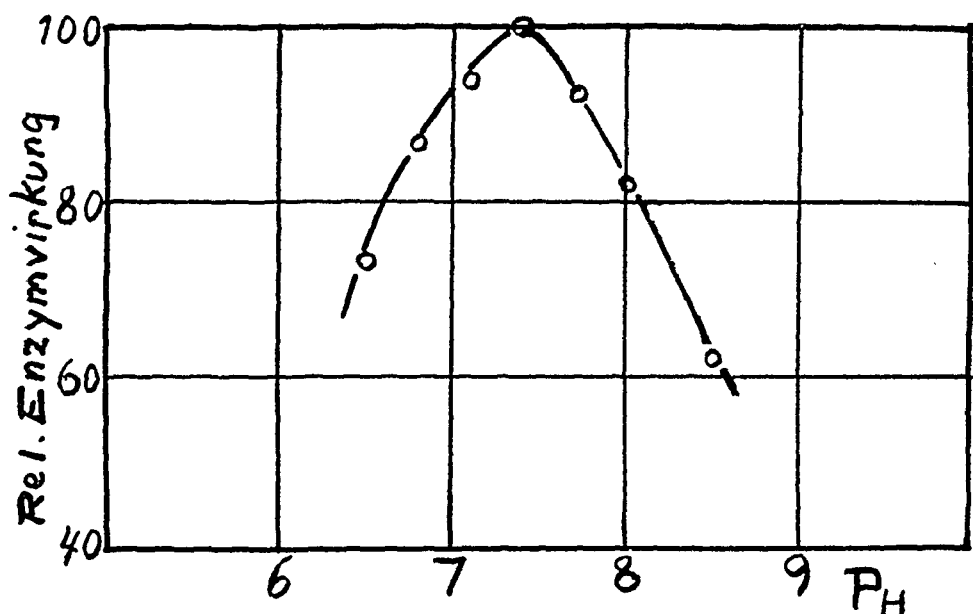


Fig. 1.

30, 60, 120 Min., werden Proben von 10 ml entnommen und in 10 ml 10proz. Trichloressigsäure pipettiert, danach wird filtriert. In 10 ml des Filtrats wird die Menge anorganischen Orthophosphat-P nach FISKE und SUBBAROW mit Hilfe eines photoelektrischen Kolorimeters bestimmt.

In einer besonderen Probe stellt man die ursprüngliche Menge anorganischen Orthophosphat-P im Blut fest.

Als Mass für die Enzymmenge berechnet man die Reaktionskonstante nach der Formel für monomolekulare Reaktion

$$K = \frac{1}{t} \log \frac{a}{a - x},$$

wo t die Anzahl der Minuten zwischen dem Beginn der Reaktion und der Zeit für die Probenentnahme bedeutet, a die totale ursprüngliche Menge Pyrophosphat-P in der Reaktionsmischung und x die während der Zeit t gesplante Menge Pyrophosphat-P. Bei der Entnahme einer andern Blutmenge als der oben vorgeschlagenen, was besonders bei starker Enzymwirksamkeit zweckmässig sein kann, rechnet man zu 1 ml Blut bzw. Plasma pro 10 ml Reaktionsmischung um.

Man kann auch in Analogie mit der von BODANSKY angegebenen Einheit für Orthomonophosphoresterase diejenige Menge

an anorganischem Orthophosphat-P feststellen, welche unter den oben angegebenen Bedingungen während 1 Stunde aus 100 ml Blut bzw. Plasma gebildet wird. Dieser Wert, der hier mit *Pyro-E* bezeichnet wird, stellt sich natürlich weniger exakt als die Reaktionskonstante, da die Spaltung nur eine kurze Zeit geradlinig verläuft, er dürfte jedoch für klinische Bestimmungen ausreichen.

Die Bestimmungen wurden mit Blut ausgeführt, dem Natriumzitrat zugesetzt war.

pH-Optimum.

KAY gibt als pH-Optimum für die Blutpyrophosphatase 7.6 und ROCHE 6.2 an. Da diese Werte ganz bedeutend divergieren, hielt ich es für nötig, dieselben zu kontrollieren. Das Resultat findet sich in Fig. 1. Als Optimum erhielt ich $\text{pH} = 7.4$, also einen Wert, der mit dem von KAY angegebenen fast übereinstimmt.

Der Pyrophosphatasegehalt im Blut.

Tabelle 1 gibt eine Zusammenfassung der erhaltenen Werte für die Pyrophosphatasewirkung, sowohl in Form von Reaktionskonstanten als auch von *Pyro-E* (in der Tabelle nur mit E bezeichnet) angegeben. Im Plasma ist die Pyrophosphatasewirkung relativ gering und zeigt bei verschiedenen Tierarten keinen statistisch sicheren Unterschied. Der Pyrophosphatasegehalt in den Erythrozyten wurde aus den Werten für Blut und Plasma mit Hilfe der Hämatokritwerte berechnet. Mit Rücksicht auf die Pyrophosphatasewirkung der Erythrozyten kann man zwei Gruppen unterscheiden. Die eine umfasst Blutkörper von Mensch, Hund und Pferd mit relativ kräftiger Wirkung, die andere Blutkörper vom Rind mit einer Enzymwirkung, die nur ungefähr $\frac{1}{10}$ der Wirkung in den erstgenannten beträgt. Es kommen jedoch sehr grosse individuelle Variationen, besonders im Rinderblut, vor. Die Werte für die Pyrophosphatasewirkung im ganzen Blut folgen nicht den Werten in den Erythrozyten, da das Blutkörpervolumen bei verschiedenen Tierarten variiert. Dasselbe ist beim Pferde mit durchschnittlich 30 am niedrigsten, beim Menschen, Hunde und Rinde findet man normale Werte zwischen 40 und 50.

In die Tabelle wurde auch das Verhältnis zwischen den Werten aufgenommen, die man nach den beiden Berechnungsmethoden

Tabelle 1.
Pyrophosphatase in Blut.

	Erythrozyten			Plasma			Blut		
	100 k	E	E	100 k	E	E	100 k	E	E
			100 k			100 k			100 k
Mann	0.84	41.8	123	0.020	3.72	186	0.92	109	118
„	0.84	43.8	129	0.014	2.34	167	0.80	103	129
„	0.42	56.8	135	0.039	5.52	142	0.95	128	135
„	0.48	62.4	130	0.028	4.48	160	1.28	165	129
Weib	0.18	21.5	120	0.010	1.64	164	0.55	66	120
„	0.19	21.4	113	0.012	2.32	193	0.53	59	111
„	0.26	32.4	125	0.014	2.24	160	0.69	86	125
„	0.31	43.0	139	0.028	4.00	143	0.88	122	138
„	0.46	50.6	110	0.014	2.32	166	1.21	133	110
Mittel	0.38	41.5	125	0.020	3.18	165	0.87	108	124
mittl. Fehl.	± 0.04			± 0.001			± 0.14		
Rel.	97	70		74	74		150	121	
Hund	0.17	19.9	117	0.022	3.52	160	0.28	33	119
„	0.36	41.7	116	0.016	3.90	200	0.65	75	115
„	0.36	48.8	136	0.041	8.00	195	0.79	103	130
„	0.49	63.1	129	0.035	5.20	148	0.90	117	130
Mittel	0.35	43.4	125	0.029	5.16	176	0.66	82	124
mittl. Fehl.	± 0.07			± 0.005			± 0.14		
Rel.	103	73		107	120		114	92	
Pferd	0.14	21.5	154	0.017	3.00	176	0.42	63	149
„	0.16	22.7	142	0.016	2.72	170	0.47	65	139
„	0.16	23.5	147	0.022	4.20	191	0.56	81	145
„	0.16	24.8	155	0.042	6.72	160	0.48	71	148
„	0.20	24.5	122	0.020	3.66	178	0.63	73	117
„	0.20	28.0	140	0.049	7.72	158	0.91	121	133
„	0.20	28.6	143	0.042	6.84	162	0.70	94	134
„	0.22	31.9	145	0.032	5.00	156	0.77	108	140
„	0.22	32.8	149	0.020	3.40	170	0.55	81	147
„	0.24	34.8	145	0.042	6.44	153	0.84	120	143
Mittel	0.19	27.3	144	0.030	4.96	166	0.64	88	138
mittl. Fehl.	± 0.01			± 0.004			± 0.05		
Rel.	56	46		111	116		110	96	
Rind	0.016	3.12	195	0.012	2.24	187	0.020	4.10	205
„	0.022	3.96	180	0.006	1.20	200	0.064	7.50	117
„	0.024	4.44	184	0.018	2.96	164	0.035	7.03	200
„	0.028	5.08	181	—	—	—	—	—	—
„	0.035	5.56	159	0.032	4.96	155	0.042	6.75	161
„	0.035	5.84	166	0.012	2.04	170	0.065	10.9	168
„	0.039	5.64	144	0.021	3.72	177	0.061	8.10	133
„	0.042	6.20	148	0.035	5.36	153	0.061	8.35	137
„	0.048	7.86	164	0.042	6.92	165	0.056	9.25	165
„	0.079	11.68	148	0.062	9.20	148	0.121	18.1	150
Mittel	0.034	5.94	167	0.027	4.30	168	0.058	8.90	160
mittl. Fehl.	± 0.006			± 0.006			± 0.010		
Rel.	10	10		100	100		10	10	

erhält. Will man nur die Werte von ein und derselben Tierart vergleichen, so liefern die Pyro-E-Ziffern wenigstens für die klinische Beurteilung brauchbare Werte. Dagegen wird der Unterschied grösser, wenn es sich um einen Vergleich zwischen der Pyrophosphatasewirkung verschiedener Grössenordnung handelt.

Die für die Pyrophosphatasewirkung in Blut und Erythrozyten erhaltenen Werte entsprechen jedoch nicht der tatsächlichen Enzymwirkung, da das Plasma — wie später gezeigt werden wird — eine hemmende Substanz enthält. Die angegebenen Werte drücken also den Einfluss aus, der in hämolysiertem Blut zur Geltung kommt. Wünscht man einen Masstab für die tatsächliche Pyrophosphatasewirkung in den Erythrozyten, so muss die Bestimmung direkt an diesen nach der Beseitigung des Plasmas ausgeführt werden. Über diese Bestimmungen wird später in dieser Mitteilung berichtet werden.

Der Pyrophosphatgehalt im Blut.

Der Pyrophosphatgehalt wird nach LOHMANN durch Erhitzung von Blut bzw. Plasma mit dem gleichen Volumen 2 n HCl 7 Minuten lang bei 100° C. bestimmt, wonach man den freigewordenen anorganischen Phosphor auf gewöhnliche Weise feststellt.

Tabelle 2.

Pyrophosphate in mg pr 100 ml.

	Anzahl Probe	Plasma		Erythrozyten	
		Variation	Mittel	Variation	Mittel
Homo . . .	6	0.10—1.17	0.70 \pm 0.13	7.26—12.60	9.81 \pm 0.97
Hund . . .	4	0.00—1.71	0.78 \pm 0.36	3.34— 7.65	5.54 \pm 1.10
Pferd . . .	9	0.10—0.56	0.31 \pm 0.05	1.90— 5.85	4.00 \pm 0.40
Rind . . .	19	0.00—0.71	0.24 \pm 0.05	2.42— 6.20	3.74 \pm 0.22

Tabelle 2 gibt die von mir erhaltenen Werte für den Pyrophosphatgehalt im Plasma und in den Erythrozyten von verschiedenen Tierarten an. Solche Bestimmungen wurden vorher beispielsweise von KERR und DAOUD ausgeführt, deren Werte im grossen ganzen mit den meinigen übereinstimmen. Offensichtlich enthält das Plasma relativ geringe Mengen an Pyrophos-

phaten, und ein deutlicher Unterschied bei verschiedenen Tierarten wurde nicht erzielt. Der durchweg niedrige Pyrophosphatasegehalt im Plasma dürfte mit der geringen Menge der daselbst vorhandenen Pyrophosphate im Zusammenhang stehen. Die Erythrozyten in Menschenblut enthalten ungefähr doppelt so viel Pyrophosphate wie die Blutkörper der übrigen Tierarten. Erythrozyten vom Menschen ergaben auch höhere Pyrophosphatasewirkung. Es liegt jedoch keine direkte Korrelation zwischen Pyrophosphatgehalt und Pyrophosphatasewirkung vor. Letztere wird offenbar durch andere Faktoren beeinflusst.

In diesem Zusammenhang kann hervorgehoben werden, dass der Gehalt an anderen organischen Phosphorsäureestern in Blutkörpern von Menschen, Hund und Pferde im Durchschnitt ungefähr 10mal höher ist als in Blutkörpern vom Rinde.

Das Verhalten der Pyrophosphatase im Blut in vitro.

Die Bestimmung der Pyrophosphatasewirkung gemäss der oben beschriebenen Methode wird in einer Reaktionsmischung ausgeführt, die im Verhältnis zum Blut hypotonisch ist, weshalb die Erythrozyten hämolysiert werden. Da die Pyrophosphatase in den Blutkörpern vorkommt, kann die Feststellung von Interesse sein, ob dieselbe ihre Wirkung auf die Reaktionsmischung auszuüben vermag, ohne dass eine Hämolysse stattfand. Dieses ist nämlich mit der Phosphormonoesterase der Fall, wie ich in einer früheren Mitteilung gezeigt habe.

Es wurden deshalb einige Versuche mit Pferdeblut ausgeführt, in dem die Pyrophosphatasewirkung auf die vorher beschriebene Weise, doch mit dem Unterschiede bestimmt wurde, dass die Reaktionsmischung ausser ihren gewöhnlichen Bestandteilen auch 0.8 Prozent NaCl enthielt. In dieser Mischung findet keine Hämolysse statt. Der Versuch wurde teils mit Blut und teils mit Blutkörperaufschlammung ausgeführt.

Letztere wurde auf die Weise hergestellt, dass nach der Zentrifugierung von Zitratblut das Plasma abpipettiert, die Blutkörper zweimal mit physiologischer Kochsalzlösung gewaschen und schliesslich in so viel physiologischer Kochsalzlösung aufgeschlammmt wurden, dass das Volumen ungefähr die Hälfte des ursprünglichen Blutvolumens betrug.

Da die Bestimmung der Pyrophosphatasewirkung in isotonischer Reaktionsmischung vorgenommen wurde, trat nur eine

Tabelle 3.

Pyrophosphatasewirkung in Pyro-E im Pferdeblut.

Blut- probe Nr.	Tage nach Blutent- nahme	Normale Reaktions- mischung	Reaktions- mischung + 0.8 % NaCl	B e m e r k u n g e n
1	0	44	8.5	Blut
2	0	52	7.5	Blut
3	0	19.8	5.5	Blut
	1	19.8	8.4	Hämolyse nahe vollständ. Hämolyse
	2	19.6	11.2	
	3	25.8	21.0	
4	0	21.5	5.9	Blut
	1	24.7	7.1	etwas Hämolyse
	2	27.5	11.3	kräftige Hämolyse
	0	3.4		Plasma
	1	1.3		
	2	11.1		
	0	62		Erythrozyten
	1	74		
	2	93		
5	0	41		Blut
	1	40		etwas Hämolyse Hämolyse
	2	41		
	3	43		
	0	5.3		Plasma
	1	6.9		
	2	6.4		
	3	8.4		
	0	114		Erythrozyten
	1	103		
	2	96		
	3	125		
6	0	34.8		Blut
	1	35.8		Hämolyse Plasma
	2	27.9		
	0	6.4		
	1	5.1		
	2	7.6		
	0	120		Erythrozyten
	1	123		
	2	96		
3a	0	175		Blutkörper.-aufschlammung
3b	0	175		Hämolyisierte >
4a	0	50	0	Blutkörper.-aufschlammung
	1	142	0	
4b	0	62		Hämolyisierte >

Blut- probe Nr.	Tage nach Blutent- nahme	Normale Reaktions- mischung	Reaktions- mischung + 0.8 % NaCl	B e m e r k u n g e n
5a	0	342	0	Blutkörper-aufschlammung
	1	422	4.6	
	2	414	14.1	etwas Hämolyse
	3	406	10.3	Hämolyse
5b	0	70		D:o + Plasma
6a	0	217	0	Blutkörper-aufschlammung
	1	261	0	
	2	393	0	Hämolyse
6b	0	70		D:o + Plasma
7	0	270		Blutkörper-aufschlammung
	1	388		
	2	992		
	3	276		Hämolyse

unbedeutende Spaltung des Natriumpyrophosphats ein, welche Spaltung dem Gehalt des Plasmas an Pyrophosphatase zuzuschreiben ist. In der Blutkörperaufschlammung liess sich unter diesen Versuchsbedingungen keine Enzymwirkung nachweisen (Tab. 3). Die Pyrophosphatase befindet sich also in den Erythrozyten in einem solchen Zustande, dass sie mit einem Substrat ausserhalb diesen nicht in Kontakt zu kommen vermag. In dieser Beziehung liegt zwischen diesem Enzym und der Phosphormonosterase ein wesentlicher Unterschied vor.

Wird die oben genannte Blutkörperaufschlammung hämolytisch und filtriert man die Stromata ab, so zeigt das Hämolysat die gleiche Aktivität wie die entsprechende Menge Blutkörperaufschwemmung in hypotonischer Reaktionsmischung. Die Pyrophosphatase ist folglich nicht an die Stromata gebunden (Tab. 3, Nr. 3 b und 4 b).

Bei der Bestimmung der Pyrophosphatasewirkung in der Blutkörperaufschlammung in hypotonischer Lösung wurden bedeutend höhere Werte als diejenigen erzielt, welche aus den Werten für Blut und Plasma berechnet wurden (Tab. 3). Dieses deutet darauf hin, dass das Plasma irgendeine Substanz enthält, die die Pyrophosphatasewirkung hemmt.

Zwecks Untersuchung dieses Verhaltens wurde folgender Versuch gemacht. Die Pyrophosphatasewirkung einer Blutkörperaufschlammung wurde auf die gewöhnliche Art bestimmt. Gleichzeitig wurde eine andere Probe mit derselben Blutkörperaufschlammung (2 ml) nebst Plasma (4 ml) angesetzt. Im letz-

teren Falle erhielt ich einen bedeutend niedrigeren Wert für die Pyrophosphatasewirkung (Tab. 3, Nr. 5 b und 6 b). *Hieraus geht also deutlich hervor, dass das Plasma eine hemmende Substanz enthält.*

Ein weiterer, hier behandelter Umstand ist die Frage, wie sich die Pyrophosphatasewirkung verändert, wenn eine Blutprobe einige Tage in vitro aufbewahrt wird, bis eine Hämolyse in derselben einsetzt.

Zwecks Untersuchung dieses Verhaltens wurde teils Pferdeblut, teils Blutkörperaufschlammung bei Zimmertemperatur (20—23° C.) 1—3 Tage lang aufbewahrt und hiernach die Phosphatasewirkung sowohl in gewöhnlicher Reaktionsmischung als auch in solcher mit Zusatz von 0.8 Proz. NaCl bestimmt.

Wurde die Bestimmung mit Blut in gewöhnlicher Lösung ausgeführt, so erhielt ich nach 2—3 Tagen, während welcher Zeit eine starke Hämolyse eingetreten war, keine sichere Änderung in der Pyrophosphatasewirkung. Dagegen nahm dieselbe etwas im Plasma zu. Bei der Bestimmung in isotonischer Lösung stieg die Pyrophosphatasewirkung entsprechend dem Grade, in welchem die Hämolyse fortschritt und ein Teil des Enzyms ins Plasma übergang.

In Blutkörperaufschlammungen nahm die Pyrophosphatasewirkung schon nach 24 Stunden zu, wenn die Bestimmung in gewöhnlicher Reaktionsmischung ausgeführt wurde (Tab. 3). Bei längerer Aufbewahrung (2—3 Tage) wurde in gewissen Fällen wieder eine Verminderung der Pyrophosphatasewirkung gefunden, was auf einer Inaktivierung des Enzyms beruhen dürfte. Beim Versuch in isotonischer Lösung wurde — wie oben genannt — in einer frischen Probe keine Enzymtätigkeit gefunden, nach 1—2 Tage langer Aufbewahrung konnte aber in einem Falle eine schwache Wirkung nachgewiesen werden. Die Ursache hierfür muss in einer beginnenden Hämolyse gelegen haben.

Die Pyrophosphatasewirkung in vom Plasma befreiten Erythrozyten.

Die Pyrophosphatasewirkung wurde in Blutkörperaufschlammungen bestimmt, die auf die oben angegebene Weise bereitet waren. Die Feststellung des Blutkörpergehalts geschah nach der Hämatokritmethode.

Tabelle 4.

Pyrophosphatase in den Erythrozyten.

	I 100 k, aus den Werten für Blut u. Plasma ber.	II 100 k, direkt bestimmt	II/I
Homo	1.28	6.95	5.4
	0.88	8.65	9.8
	0.95	9.20	9.7
	Rel. Mit. 15	60	Mittel 8.3
Hund	0.90	2.13	2.4
	0.65	2.54	3.9
	Rel. Mit. 11	17	Mittel 3.2
Pferd	0.48	8.65	18
	0.91	10.50	12
	0.70	12.20	17
	Rel. Mit. 10	76	Mittel 16
Rind	0.061	0.112	1.8
	0.042	0.124	3.0
	0.061	0.132	2.2
	0.121	0.178	1.5
	Rel. Mit. 1	1	Mittel 2.1

In Tabelle 4 finden sich die für die Pyrophosphatasewirkung in den Erythrozyten erhaltenen Werte, die teils aus den Werten für Blut und Plasma berechnet, teils direkt in der Blutkörperaufschlammung bestimmt wurden. Die letzteren Werte wurden dahin umgerechnet, dass sie für das gleiche Blutkörpervolumen wie die erstgenannten gelten, also 4 ml auf 40 ml Reaktionsmischung.

In sämtlichen Blutarten erhielt ich nach der letztgenannten Methode bedeutend höhere Werte. Bei Rinder-, Hunde und Menschenblutkörpern belief sich die Steigerung abgerundet auf das 2-, 3- bzw. 8-Fache, bei Pferdeerythrozyten war die Wirkung 16mal stärker. Hieraus geht hervor, dass das Pferdeblut besonders reich an dem hemmenden Stoff ist.

Der Gehalt der Erythrozyten an Pyrophosphatase bei dem Menschen und verschiedenen Tierarten variiert ganz bedeutend und gibt für die untersuchten Fälle steigende Werte in der Reihenfolge: Rind, Hund, Mensch, Pferd.

Zusammenfassung.

1. Eine Methode zur Bestimmung der Pyrophosphatase-wirkung in Blut bezw. Plasma, Serum und Erythrozyten wird beschrieben.

2. Das pH-Optimum für die Pyrophosphatasewirkung im Blut liegt bei 7.4.

3. Die Pyrophosphatasewirkung wurde im Blut und Plasma bei Mensch, Hund, Pferd und Rind bestimmt. Mit Hilfe dieser Werte und der Hämatokritwerte wurde die Wirkung in den Erythrozyten berechnet. Blut der beiden erstgenannten enthält ungefähr 10mal mehr Pyrophosphatase als Rinderblut, und Pferdeblut ungefähr 5mal so viel. Die Pyrophosphatase kommt hauptsächlich in den Erythrozyten vor, Plasma enthält nur unbedeutende Mengen.

4. Wird die Pyrophosphatasewirkung direkt in vom Plasma befreiten Erythrozyten bestimmt, so erhält man bedeutend höhere Werte als bei der Berechnung der Wirkung aus den Werten für Blut und Plasma. Die Zunahme variiert zwischen dem Doppelten und 18-Fachen. Die Enzymwirkung in den Erythrozyten verhält sich bei Pferd, Mensch, Hund und Rind wie 76 : 57 : 17 : 1.

5. Irgendeine Korrelation zwischen dem Gehalt der Blutkörper an Pyrophosphaten und Pyrophosphatase liegt nicht vor.

6. Wird die Bestimmung der Pyrophosphatasewirkung in einer Reaktionsmischung ausgeführt, die mit den Blutkörpern isotonisch ist, so erhält man keine Enzymwirkung. Die Pyrophosphatase in den Blutkörpern kann folglich nicht ausserhalb diesen zur Wirkung gelangen, bevor die Erythrozyten hämolytisch sind. Hierin unterscheidet sich die Pyrophosphatase von der Phosphormonoesterase.

7. Plasma bezw. Serum enthält einen Stoff, der die Pyrophosphatasewirkung stark hemmt. Dieser Umstand erklärt das in Punkt 4 genannte Verhalten.

8. Bei der Aufbewahrung von Blutproben bei ungefähr 20° C. für eine Zeit bis zu 3 Tagen ändert sich die Pyrophosphatasewirkung nur unbedeutend. In vom Plasma befreiten Blutkörperaufschlammungen nimmt die Pyrophosphatasewirkung nach 24 Stunden langer Aufbewahrung bei 20° C. zu.

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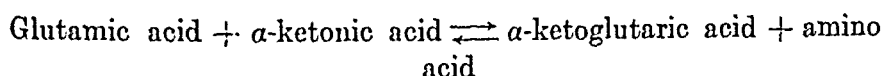
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Transamination with Peptide Substrates in Cattle Diaphragm Muscle.¹

By

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The disappearance of added glutamic in pigeon breast muscle was first discovered by NEEDHAM (1930), while the concentration of van Slyke nitrogen remained stable. From the results obtained she concluded that the amino group of glutamic acid is transferred to a reactive carbohydrate compound in order to form a new amino acid. BRAUNSTEIN and KRITZMANN (1937, 1938, 1939) found the explanation in the reaction:



which was isolated and examined.

The reaction is reversible and speedy under certain conditions. It has been found in most animal tissues and the reaction is termed "transamination" (BRAUNSTEIN and KRITZMANN, 1937). The frequency of transamination and the rapid rate at which it occurs suggest the reaction playing a prominent part in intermediary tissue metabolism, the significance of which being as yet not determined. In the present paper the problem is attacked from a different angle: The possible formation of dipeptides in a reaction between glutamic acid and α -ketonic acids in peptide linkage with an amino acid. A reaction including the disappearance of Van Slyke nitrogen in liver extracts has been scrutinized in a series of experiments (ÅGREN c. s. 1937, 1939; ÅGREN, 1940 a).

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One of the initial steps in the process was apparently a reaction between amino and aldehyde groups (amino acids and glucose), in which compounds were formed similar in type to the Schiff bases. The theory was advanced that, in a reaction between ketoaldehydes and amino acids, a compound could be obtained which would develop into a peptide after dehydrogenation and transamination. The rapid interaction between methylglyoxale and amino acids was demonstrated in a recent paper (ÅGREN 1940 b). In the present paper one of the conclusive steps is described by help of experiments, i. e. the transamination of ketonic acid-amino acid compounds.

Procedure and Methods.

General procedure. In studying the transamination it is expedient that this reaction is separated from others comprising amino and α -ketonic acids. Many of these lateral reactions require molecular oxygen and could, therefore, eventually be eliminated by working under anaerobic conditions. According to rule the results obtained by this method should not differ from those acquired aerobically in the presence of bromoacetate and sodium arsenite (BRAUNSTEIN and KRITZMANN, 1937). These substances are inhibitors preventing certain lateral reactions. Neither of these methods could completely eliminate the lateral reactions in cattle muscle, as glutamic acid disappeared slowly, anaerobically as well as aerobically, in the presence of bromoacetate and sodium arsenite, without any addition of ketonic acids. This lateral reaction may be due to transamination of free or bound ketonic acids provided by the muscle. The rate of reaction is, however, so slow that it does not interfere with the transamination of added ketonic acids.

A study of transamination was made by the determination of glutamic acid, formed either by adding α -ketoglutaric acid, amino acids and dipeptides, or made to disappear by adding glutamic acid and free or bound α -ketonic acids to muscle suspensions. In each experiment controls were made with α -ketoglutaric acid or glutamic acid alone in order to distinguish the unspecific formation of glutamic acid from preformed amino group donators (NH_3 and amino acids) or the consumption of glutamic acid by preformed ketonic acids. The procedure taken by BRAUNSTEIN and KRITZMANN (1937) in the determination of glutamic acid is

not specific and other controls were necessary. In many of the transamination experiments the glutamic acid precipitate is contaminated with some of the amino group donating substrates (amino acids, peptides). In all experiments, therefore, blank determinations were carried out by adding amino group donators and receptors to the heat inactivated muscle suspension. Tests were also made without adding donator and receptor substances. A considerable variation was apparent in the activity of the transamination system in muscle suspensions from different animals. Consequently the activity of the transamination enzymes in the experiments with peptides and α -ketonic acid-amino acid compounds were controlled by following the reaction between alanine and α -ketoglutaric acid or pyruvic acid and glutamic acid.

Experimental procedure. Cattle diaphragm muscle was chilled in the slaughter-house immediately after the death of the animal, and finely cut in a mincer. The minced muscle was suspended in 4 parts of 0.2 per cent KHCO_3 , the substrates and inhibitors being added as neutralized solutions. Usually 100—200 μ Mol. of substrate per gram of muscle was added. Substrates such as glycyl-aminobenzoic acid, soluble with difficulty, were added in solutions saturated at 40° to muscle suspensions of the same temperature. The experiments were carried out in Thunberg tubes, anaerobic or aerobic conditions being maintained by filling the vessels with N_2 or O_2 . The tubes were shaken at 40° for 30 minutes, unless otherwise stated. In the space of time mentioned the transamination was complete. At the end of the experimental period the solutions were speedily heated to 100° and then chilled again. Trichloroacetic acid was added to a final concentration of 5 per cent.

Analytical procedure. Glutamic acid was determined by the JONES and MOELLER method (1928) in the modification of BRAUNSTEIN and KRITZMANN (1937), 3 cc of trichloroacetic acid filtrate being used in each analysis. The values are expressed as mg van Slyke nitrogen in glutamic acid per cc filtrate. Ammonia was determined according to CONWAY and BYRNE (1933), total amino nitrogen by the van Slyke method and α -ketonic acids by titration with cerium sulphate (FROMAGEOT and DESNUELLES, 1933). Ketonic acid was calculated from the titration values as mg/cc of α -ketoglutaric acid or pyruvic acid, depending on the substrate used. α -ketoglutaric acid was synthesized according to the NEUBERG and RINGER method (1915).

Results. The reaction between α -ketoglutaric acid and alanine and the reversibility of this reaction was first investigated. The results of one of the series of experiments are given in table 1. Muscle from one animal was used in each series of experiments. The transaminating enzyme is called aminopherase in accordance with BRAUNSTEIN and KRITZMANN (1939).

Table 1.

Anaerobic transamination with glutamic acid + pyruvic acid and α -ketoglutaric acid + alanine.

Substrate added	Total amino-N in mg/cc		Amino-N in glutamic acid fraction in mg/cc		Δ in glutamic acid in mg/cc	Ketonic acid in mg/cc	
	0 min.	30 min.	0 min.	30 min.		0 min.	30 min.
α -ketoglutaric acid + alanine	0.71	0.71	0.17	0.30	+ 1.37	3.1	3.0
α -ketoglutaric acid . .	0.16	0.71	0.072	0.084	+ 0.13	3.1	3.1
Alanine	0.71	0.71	0.108	0.097	- 0.11	0.2	0.2
Blank	0.16	0.17	0.068	0.070	+ 0.02	0.3	0.2
Glutamic acid + pyru- vic acid	0.32	0.32	0.24	0.19	- 0.53	3.2	3.1
Glutamic acid	0.31	0.31	0.20	0.19	- 0.10	0.3	0.2

3 g. muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. Glutamic acid 2 mg/cc. + pyruvic acid 3 mg/cc. α -ketoglutaric acid 3 mg/cc + 1 (+) - alanine 3.6 mg/cc.

The reversibility of the transamination is clearly demonstrated. Starting with alanine and α -ketoglutaric acid, the glutamic acid formation corresponded to a transamination of 21 per cent of added NH_2 -nitrogen. In the opposite direction 22 per cent of the added glutamic acid was lost. The character of a true equilibrium is not demonstrable, since the plateaux reached from both sides of the reaction lie at different levels. It may here be noted that the end point (or difference in glutamic acid) was independent of the absolute amounts of reacting substrates. Starting with 50 μMol . glutamic acid or 100 μMol . α -ketoglutaric acid per gram of muscle, and with a surplus of pyruvic acid or alanine in both experiments, the result obtained was a 20 per cent reaction. There was no change in total van Slyke nitrogen, nor any loss of ammonia. In contrast to the results obtained by BRAUNSTEIN and KRITZMANN

(1937) the concentration of ketonic acids was unchanged. Ketoglutaric acid seemed less receptive towards lateral reactions in cattle muscle than in pigeon breast muscle.

Possibilities of reaching an equilibrium in the reaction:

glutamic acid + pyruvic acid \rightleftharpoons α -ketoglutaric acid + alanine

appeared to vary in muscles of different animals. BRAUNSTEIN and KRITZMANN (1937) reported the equilibrium constant to be 1. In the experiments with cattle muscle usually about 30 to 50 per cent of the added glutamic acid disappeared; only 5 experiments of 40 were negative. In the opposite direction (α -ketoglutaric acid + alanine) the results varied considerably. In the attempts to elucidate this irregularity in the reaction the influence of freezing the muscle was examined. The muscle was chilled as usual in the slaughter-house with a salt-ice mixture for the transport to the laboratory, the whole proceeding taking about 15 minutes. On arrival at the laboratory the muscle usually had a temperature of $+2^{\circ}$. After mincing a part of the muscle was immediately taken to experiments, the rest of the minced muscle was frozen at -20° for 3 hours. A comparison of transamination in the unfrozen and the frozen muscle is given in table 2.

Table 2.

Anaerobic transamination in unfrozen and frozen muscles.

Substrates added	Muscle before experiment	Total amino-N in mg/cc		Amino-N in glutamic acid fraction in mg/cc		Δ in glutamic acid in mg/cc	Ketonic acid mg/cc	
		0 min.	30 min.	0 min.	30 min.		0 min.	30 min.
Glutamic acid + pyruvic acid	unfrozen	0.58	0.59	0.34	0.26	- 0.84	3.9	4.0
	frozen	0.58	0.58	0.34	0.27	- 0.74	4.1	4.0
Glutamic acid.	unfrozen	0.57	0.58	0.31	0.30	- 0.10	0.2	0.3
α -ketoglutaric acid + alanine	unfrozen	0.69	0.70	0.21	0.23	+ 0.21	3.1	3.2
	frozen	0.70	0.70	0.17	0.25	+ 0.84	3.0	3.1
α -ketoglutaric acid	frozen	0.15	0.16	0.07	0.08	+ 0.10	2.9	3.0
Blank	frozen	0.15	0.15	0.06	0.06	—	0.2	0.3

3 g. muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. Glutamic acid 3 mg/cc + pyruvic acid 4 mg/cc. α -ketoglutaric acid 3 mg/cc + 1 (+) - alanine 3.6 mg/cc.

Several experiments, all with results pointing in the same direction as the values in table 2, emphasize the conclusion that the reaction between glutamic acid and pyruvic acid is not considerably influenced by freezing the muscle. On the other hand the reaction between α -ketoglutaric acid and the NH_2 -donators (alanine and peptides) is decidedly stronger after freezing the muscle. By this procedure the latter reaction is brought up to the same level as the reaction between glutamic acid and pyruvic acid. It is most probable that some factor in the reaction between α -ketoglutaric acid and alanine would be more easily extractable by preliminary freezing. There was no influence noted on the total van Slyke nitrogen neither on the ketonic acid concentration by this procedure. In the following frozen muscles were always used in reactions between α -ketoglutaric acid and NH_2 -donators.

A comparison was also made between transamination in cattle muscle suspensions in aerobic and anaerobic conditions. The results obtained from a series of experiments are given in table 3.

Table 3.

Transamination during anaerobic conditions.

Substrates added		Amino-N in glutamic acid fraction in mg/..		Δ in glutamic acid fraction in mg/cc	α -ketoglutaric acid in mg/cc	
		0 min.	30 min.		0 min.	30 min.
Glutamic acid + pyruvic acid	anaerobic	0.38	0.25	- 0.74	3.9	4.0
	aerobic	0.33	0.25	- 0.84	4.0	4.0
Glutamic acid	anaerobic	0.33	0.29	- 0.42	0.3	0.2
	aerobic	0.33	0.29	- 0.42	0.3	0.3
α -ketoglutaric acid + alanine	anaerobic	0.17	0.27	+ 1.05	2.9	3.0
	aerobic	0.17	0.29	+ 1.26	3.0	3.0
α -ketoglutaric acid	anaerobic	0.18	0.22	+ 0.42	3.1	3.0
	aerobic	0.17	0.22	+ 0.52	3.0	2.9

3 g. frozen muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. Glutamic acid 3 mg/cc. + pyruvic acid 4 mg/cc. α -ketoglutaric acid + 3 mg/cc + alanine 3.6 mg/cc.

It is clearly demonstrated that the results gained aerobically are essentially the same as those gained anaerobically. If anything, these experiments show that the formation and the disappearance of glutamic acid are slightly higher under aerobic conditions. The

effect of adding inhibitors, bromoacetate and sodium-arsenite, was also investigated. These inhibitors were recommended by BRAUNSTEIN and KRITZMANN (1937) in aerobic experiments to separate the transamination reaction from other lateral reactions which could interfere with the quantitative results. A typical series of an experiment is listed in table 4.

Table 4.

The effect of bromoacetate and sodium arsenite on aerobic transamination.

Substrate added	Inhibitor	Amino-N in glutamic acid fraction in mg/cc.		Δ in glutamic acid in mg/cc.	Ketonic acids in mg/cc.	
		0 min.	30 min.		0 min.	30 min.
Glutamic acid + pyruvic acid	+	0.34	0.25	- 0.95	3.9	4.0
	0	0.34	0.26	- 0.84	4.1	4.0
Glutamic acid	+	0.33	0.29	- 0.42	0.2	0.3
	0	0.33	0.29	- 0.42	0.3	0.3
α -ketoglutaric acid + alanine	+	0.09	0.25	+ 1.65	3.0	3.0
	0	0.17	0.32	+ 1.57	3.1	3.1
α -ketoglutaric acid	+	0.10	0.14	+ 0.42	3.0	3.1
	0	0.15	0.19	+ 0.42	3.1	3.1

3 g. frozen muscle + 1.2 cc. of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. Glutamic acid 5 mg/cc. + pyruvic acid 4 mg/cc. α -ketoglutaric acid 3 mg/cc + alanine 3.6 mg/cc.

The addition of bromoacetate and sodium arsenite does not influence the transamination level. If anything, the transamination was more effective when the inhibitors were added. Under such circumstances as these the following experiments were carried out aerobically with frozen muscles and in the presence of sodium arsenite and bromoacetate.

Transamination with Peptides.

In working with peptides as NH_2 -donators one of the difficulties met with was the hydrolyzing effect of the muscle peptides. In a preceding paper (ÅGREN, 1940 c) it was demonstrated how at least two di-peptides, glycyl-aminobenzoic acid and valyl-glycine, were but slowly attacked by the peptidases in the cattle muscle.

The experimental conditions were the same as those demonstrated above, so as to be optimal in transamination. A typical experiment with glycyl-aminobenzoic acid is recorded in table 5.

Table 5.

Glutamic acid formation from α -ketoglutaric acid + glycyl-aminobenzoic acid. Aerobic experiments.

Substrates added	Total amino-N in mg/cc		Amino-N in glutamic acid fraction in mg/cc			Δ in glutamic acid fraction in mg/cc
	0 min.	60 min.	0 min.	20 min.	60 min.	
Glycyl-aminobenzoic acid + α -ketoglutaric acid . . .	0.54	0.54	0.12	0.14	0.15	+ 0.32
α -ketoglutaric acid	0.18	0.18	0.07		0.08	+ 0.10
alanine + α -ketoglutaric acid	0.72	0.72	0.18		0.26	+ 0.84

3 g. frozen muscle + 1.2 cc of 2 per cent of KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. α -ketoglutaric acid 3 mg/cc + glycyl-aminobenzoic acid 5 mg/cc, alanine 3.6 mg/cc.

Two facts were observed when comparing the peptide and the amino acid reaction in many experiments. The peptide NH_2 -donator was slower in its reaction and the transamination did not proceed to the same extent as with the amino acid donator. Only after 60 minutes did the reaction with glycyl-aminobenzoic acid cease. Valyl-glycine appeared to be a more easily reacting peptide, as demonstrated in table 6.

Table 6.

Glutamic acid formation from α -ketoglutaric acid and valylglycine. Aerobic conditions.

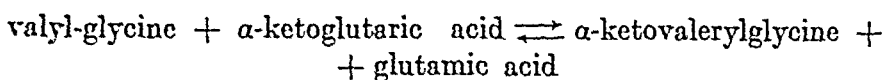
Substrates added	Total-N in mg/cc		Amino-N in glutamic acid fraction in mg/cc		Δ in glutamic acid in mg/cc
	0 min.	15 min.	0 min.	15 min.	
α -ketoglutaric acid + valylglycine .	0.71	0.72	0.11	0.17	+ 0.69
α -ketoglutaric acid	0.17	0.17	0.08	0.09	+ 0.10
α -ketoglutaric acid + alanine . . .	0.71	0.72	0.18	0.25	+ 0.73

3 g. frozen muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. α -ketoglutaric acid 3 mg/cc + valylglycine 7 mg/cc, alanine 3.6 mg/cc.

The effectivity of valyl-glycine as NH_2 -donator is clearly demonstrated. The shorter time of reaction (15 min.) was adapted in the experiments with valyl-glycine, as preliminary experiments had shown that exceptionally slight hydrolysis of valyl-glycine could be traced after 30 minutes of incubation. The reaction with valyl-glycine was of the same order of magnitude as with alanine.

About 30 per cent of added NH_2 -donators were consumed. The total van Slyke nitrogen values were constant during the experiments, indicating that valyl-glycine was transformed to α -keto-valeryl-glycine.

Experiments were also carried out to test the reversibility of the reaction:



BRAUNSTEIN and KRITZMANN reported (1937) that lactic acid could act as acceptor in transamination experiments. They suggested a preliminary dehydrogenation of lactic acid to pyruvic acid. A test was made to discover whether a similar mechanism existed in cattle muscle and could be used for transamination in the above-mentioned reaction. The corresponding α -hydroxy-valeryl-glycine was prepared from α -brom-valeryl-glycine. In a similar way α -hydroxypropionyl-glycylglycine was obtained. A few results with these substrates are given in table 7.

Table 7.

Transamination with α -hydroxyacids in peptide linkages as acceptors.

Substrates added	Total amino-N in mg/cc		Amino-N in glutamic acid fraction in mg/cc		Δ in glutamic acid in mg/cc	Ketonic acids in mg/cc	
	0 min.	30 min.	0 min.	30 min.		0 min.	30 min.
Glutamic acid + pyruvic acid	0.50	0.51	0.33	0.26	- 0.68	4.1	4.1
Glutamic acid	0.51	0.51	0.29	0.29	—	0.2	0.3
Glutamic acid + lactic acid	0.49	0.49	0.28	0.27	- 0.10	0.3	0.3
Glutamic acid + α -hydroxyglycylglycine .	0.45	0.45	0.28	0.55	- 0.32	0.3	0.4
Glutamic acid + α -hydroxyvaleryl-glycine .	0.46	0.46	0.28	0.26	- 0.21	0.4	0.4

3 g. frozen muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. Glutamic acid 3 mg/cc + pyruvic acid 4 mg/cc, lactylglycylglycine 7.6 mg/cc, lactic acid 3.6 mg/cc. α -hydroxyvaleryl-glycine 7 mg/cc.

There seemed to be a tendency towards α -hydroxyacids in peptide linkage with an amino acid functioning as NH_2 -acceptors. Experiments with the corresponding α -ketonic acids met with difficulties in the preparation. The problem was attacked in an indirect way. α -ketoglutaric acid and the NH_2 -donator (amino acid or peptide) were brought to reaction in the muscle suspension. Then the reaction was forced back again by adding a surplus of glutamic acid. The result of a typical series is given in table 8.

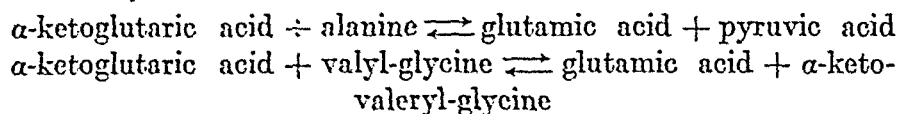
Table 8.

The reversibility of transamination with amino acids and peptides. Aerobic conditions.

Substrates added	Total amino-N in mg/cc			Amino-N glut- amic acid frac- tion in mg/cc			Δ in glutamic acid in mg/cc		Ketonic acids in mg/cc		
	0 min.	20 min.	30 min.	0 min.	20 min.	30 min.	20 min.	30 min.	0 min.	20 min.	30 min.
α -ketoglutaric acid + alanine = Co_{20} .	0.45	0.46	—	0.13	0.17	—	+ 0.42	—	3.0	3.1	—
α -ketoglutaric acid + alanine. After 20 min. stopped im- mediately on the addition of glut- amic acid = GIA_{20}	0.45	0.63	—	—	0.39	—	—	—	3.1	3.1	—
As GIA_{20} but stopp- ed 10 min. after adding glutamic acid = GIA_{30} . .	0.45	—	0.64	—	0.39	0.37	—	- 0.21	—	—	3.2
α -ketoglutaric acid	0.15	—	0.14	0.06	—	0.07	—	+ 0.10	3.1	—	3.2
α -ketoglutaric acid + valylglycine . .	0.71	0.72	—	0.16	0.27	—	+ 1.12	—	3.2	3.1	—
As GIA_{20} but valyl- glycine substitut- ing alanine . . .	—	0.90	—	—	0.42	—	—	—	—	3.2	—
As GIA_{30} but valyl- glycine substitut- ing alanine . . .	—	—	0.91	—	—	0.37	—	- 0.53	—	—	3.1
Glutamic acid 2 mg/cc	0.31	—	0.81	0.20	—	0.19	—	- 0.10	0.3	—	0.2

3 g. frozen muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. α -ketoglutaric acid 3 mg/cc + alanine, 1.8 mg/cc. After 20 min. incubation addition of glutamic acid to a concentration of 2 mg/cc. Valylglycine 7 mg/cc.

The table demonstrates the possibilities of influencing the stationary level in the two reversible reactions:



Starting from left to right, the end point obtained can be forced back a little to the left by adding glutamic acid. Thus it appears reasonable to assume that α -ketovaleryl-glycine can function as NH_2 -acceptor to form a dipeptide. The assumption is emphasized by the constancy of the total van Slyke nitrogen values.

The difference in reactivity of the peptides and amino acids raised the question of which substrates could function together with α -ketoglutaric acid to form glutamic acid. In the first series of experiments, quoted above, a l(+)-alanine preparation was used. Being without this preparation for a time, a d, l-alanine, in a concentration twice as high, was added. The yield of glutamic acid in the latter series was much higher than expected. Consequently, the optical isomers and the racemic compounds of a few amino acids were examined. The results of these experiments are listed in table 9.

Table 9.

Glutamic acid formation from α -ketoglutaric acid and different amino acids.

Amino acids added	Number of experiment	Amino-N in glutamic acid fraction in mg/cc		Δ in glutamic acid in mg/cc
		0 min.	30 min.	
l(+) alanine	1	0.11	0.17	+ 0.68
d, l alanine	1	0.17	0.27	+ 1.05
l(+) alanine	2	0.14	0.22	+ 0.84
d(−) alanine	2	0.14	0.17	+ 0.32
d, l alanine	2	0.12	0.21	+ 0.95
l(+) valine	2	0.07	0.11	+ 0.42
d(−) valine	2	0.09	0.11	+ 0.21
d, l valine	2	0.08	0.12	+ 0.42

Experiments with the identical numbers carried out with muscle suspensions from the same muscle. The values corrected for the spontaneous reaction of α -ketoglutaric acid. 3 g. frozen muscle + 1.2 cc of 2 per cent KHCO_3 + water; total volume 15 cc. Sodium arsenite m/100, bromoacetate 1:5000. α -ketoglutaric acid 3 mg/cc, l(+) and d(−)-alanine 1.8 mg/cc. d, l-alanine 3.6 mg/cc. l(+) − and d(−)-valine 2 mg/cc, d, l-valine 4 mg/cc.

A number of experiments were made, all varying in the same way in the results. Cattle muscle seems to have a tendency towards forming glutamic acid from the unnatural forms of amino acids, but this tendency varies in different muscles.

Discussion. It has been proved in the first section of the present paper that cattle diaphragm muscle can be used as a reliable resource for enzymes, when working at transamination experiments. This is of some practical interest, as this muscle can be obtained in great amounts immediately after the death of the animals. BRAUNSTEIN's and KRITZMANN's results (1937) regarding the formation of glutamic acid from α -ketoglutaric acid and alanine have been corroborated. On the other hand a discrepancy was discovered in the reaction of ketonic acids. The Russian workers found that ketonic acids disappeared during the transamination. This result was interpreted as a transformation of α -ketoglutaric acid to succinic acid. It is evident that this lateral reaction, including the decarboxylation of α -ketoglutaric acid, is not as apparent in cattle muscle as in pigeon breast muscle. There may be some variation in the activity or concentration of the decarboxylating system, according to how the requirement and metabolism in carbohydrate varies. On the other hand this variation may be a secondary one, originating in the release of otherwise structurally connected enzymes.

It is of interest to observe that α -keto groups in peptide linkages to amino acids can function as acceptors of amino groups and form dipeptides. The part played by such a mechanism in protein metabolism will not be discussed in this connection, but it has been remarked before that such compounds could be formed in a reaction between ketoaldehydes and amino acids. The varying results in the experiments with the optical isomers of amino acids may be of interest in the light of KÖGL's results (1939). An individually varying activity in the transformation of unnatural forms of amino acids is a possibility worth paying attention to.

The author wishes to express his gratitude to Professor K. LINDERSTRÖM-LANG for extending the facilities of the Carlsberg Laboratory and for his untiring interest in the work.

Summary.

1. Transamination has been studied in cattle diaphragm muscle. The reaction proved, with slight discrepancies, the well known characteristic properties.

2. The reaction between α -ketoglutaric acid and amino acids or peptides was facilitated by preliminarily freezing the minced muscle. A disappearance of ketonic acids could not be demonstrated. The transamination was not inhibited by bromoacetate or sodium arsenite.

3. Glycyl-aminobenzoic acid and valyl-glycine could be used as amino group donators. In the opposite way glutamic acid apparently reacted with α -hydroxy and α -ketonic acids in peptide chains.

4. Muscles from different animals displayed some variability in the reaction between α -ketoglutaric acid and unnatural amino acids and racemic derivatives.

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The Absorption of Ethyl Alcohol from the Gastro-Intestinal Tract as a Diffusion Process.¹

By

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(With 4 figures in the text.)

Introduction.

Previous investigations on the distribution of alcohol in the system indicate that this process must follow certain laws (GRÉHANT 1895, CARPENTER 1929, WIDMARK 1930 and NICLOUX 1934), and a number of investigators have been able to prove that its passage to various fluids in the body takes place by diffusion (urine: WIDMARK 1916, milk: OLOW 1923, cerebrospinal fluid: ABRAMSON and LINDE 1930, gall: NICLOUX 1931, saliva: LINDE 1932, sweat: NYMAN and PALMLÖV 1936, aqueous humour: BERGGREN). There is therefore reason to assume that the same process applies to the absorption from the gastro-intestinal tract. There are, however, very few references to support this assumption clearly.

Direct examinations of the contents of the stomach and the intestines show that all parts of the alimentary canal are capable of absorbing alcohol (TAPPEINER 1880, NEMSER 1907, HANZLIK and COLLINS 1913, LUCAS 1930, CARPENTER 1937). In some cases the alcohol percentage has been determined in the contents of the stomach or the intestines running out of fistulae (KAUTSCH 1898, LÖNNQUIST 1906, NEMSER 1907), in some instances by

¹ Received 6 September 1940.

direct determination of the contents when withdrawn from the stomach after ligating the pylorus or from isolated loops of the intestines (TAPPEINER 1880, SEGALL 1888, BRANDL 1893, HANEBORG 1921, EDKINS and MURRAY 1926) and lastly by analysing the contents after killing different animals at different intervals (VÖLTZ and DIETRICH 1915, CORI, VILLIAUME and CORI 1930, LÉ BRETON 1936, HARGER, HULPIEU and LAMB 1937). These investigations are, however, incomplete to a certain extent and do not give absolutely comparable results, in consequence of which it has not been possible to obtain a true picture of the changes that the alcohol percentage undergoes in the contents of the stomach and the intestines; generally speaking, however, it seems as if the alcohol is more quickly absorbed during the first hour of the experiment and also to a fair extent already in the stomach.

Several investigators have also endeavoured to come to some conclusions concerning the course of absorption from the point of the blood alcohol curve:

When alcohol is ingested on an empty stomach absorption seems to take place comparatively quickly and to be completed in 1 or 2 hours (MELLANBY 1919, WIDMARK 1932, BERNHARD and GOLDBERG 1935), others maintain that it takes 5—6 hours (HAGGARD and GREENBERG 1935). A stronger percentage of the alcohol ingested seems to bring about a more rapid absorption (MELLANBY 1919, MILES 1922), though HANDWERK (1927) and TUOVINEN (1930) contradict this, while other investigators have not been able to find any difference (WIDMARK 1932, SCHMIDT 1937). Absorption seems to take place more quickly in those habituated to alcohol than in normal individuals (humans: SCHWEISHEIMER 1913, JUNGMICHEL 1933, SCHMIDT 1934, FLEMING and STOTZ 1935, BERNHARD and GOLDBERG 1935, animals: VÖLTZ and DIETRICH 1915, FAURE and LOEWE 1923, NEWMAN and CARD 1937), something that has not been confirmed by some investigators (humans: MILES 1922, MATOSSI 1931, GRAF and FLAKE 1932, animals: GETTLER and FREIREICH 1935). — Simultaneous ingestion of food delays absorption, and some authors look upon the constituent parts of the food as playing a certain rôle (MELLANBY 1919, SOUTHGATE 1925, WIDMARK 1934, NEYMARK and WIDMARK 1936, SCHMIDT 1937), other investigators take the caloric percentage into consideration (JUNGMICHEL 1933), while ELBEL and LIECK (1936) attach importance to the quantity.

From the literature on the absorption of alcohol it may be summarily concluded that ethyl alcohol is obviously absorbed with gradually diminished rapidity according to the period of time from the beginning of the absorption. The greater quantity of alcohol is absorbed during the first hour, unless large doses have been given, but in particular cases considerable unabsorbed quantities may be found even after several hours. The concentration of the alcohol solution has been assigned different significance in different experiments. The simultaneous ingestion of food produces a delay in the absorption, but to what extent this may be regarded as having a diluting effect has, according to our opinion, hitherto received too little consideration.

Own Investigations.

Mathematical Analysis.

The object of this investigation was to follow the change in the concentration of ethyl alcohol in the contents of the stomach during absorption by means of direct determinations and to see whether there is any criterion for the assumption that the absorption is a diffusion process. Before describing the methods and their results, a general analysis is given of the factors influencing the passage of alcohol from the gastro-intestinal tract to the system. For this analysis it must be assumed that alcohol diffuses freely through all the tissues of the body and that the concentration when equilibrium has been established is fixed by the percentage of water in the different tissues.

Assuming a pure diffusion, the factors establishing the disappearance of alcohol from the contents of the stomach are as follows:

The concentration of the ingested alcoholic solution.

The motility of the gastric wall (the mixing up of the contents).

The permeability of the gastric mucous membrane.

The number of capillaries in the mucous membrane.

The blood flow through the mucous membrane.

According to FICK's law of diffusion, the rapidity of diffusion is directly proportional to the difference of the concentration between the two media (in this case the contents of the stomach and the blood). On account of the alcohol percentage in the blood during the greater part of the absorption being slight in comparison with the percentage of alcohol in the contents of the stomach, the former may be ignored. Expressed in mathematical form, the process of diffusion may be signified

$$-\frac{dc}{dt} \cdot V = a \cdot c \quad (1)$$

Thus $\frac{dc}{dt}$ gives the change in concentration pr time unit (— sign gives decrease in concentration),

V = quantity of alcoholic solution,

a = diffusion constant,

c = concentration of alcoholic solution.

Development of above formula gives

$$-\frac{dc}{c} = \frac{a}{V} \cdot dt;$$

Integration

$$\ln c = -\left(\frac{a}{V} \cdot t + k\right)$$

$$c = e^{-\frac{a}{V} \cdot t} \cdot e^{-k};$$

When

$$t = 0; c = e^{-k};$$

$$c = c_0; e^{-k} = c_0.$$

Thus

$$c = c_0 \cdot e^{-\frac{a}{V} \cdot t} \dots \dots \dots (2)$$

The quantity of alcohol in the tissues of the body on a given occasion (t) may then be defined as follows:

$$V \cdot c_0 - V \cdot c_t - \beta \cdot r \cdot t \cdot p$$

β = rate of disappearance of alcohol from the blood in promille pr min,

r = relation between percentage of alcohol in the body and in the blood,

t = time in min,

p = body weight in kg.

After replacing of c_t according to formula (2)

$$V \cdot c_0 - V \cdot c_0 \cdot e^{-\frac{a}{V} \cdot t} - \beta \cdot r \cdot t \cdot p \dots \dots \dots (3)$$

If V and c_0 in formula (3) are allowed to vary in an opposite direction, *i. e.* to allow the absolute quantity of alcohol ingested to be identical but to let the quantity of the solution ingested and the concentration vary, it will obviously bring about changes in the total quantities of alcohol existing in the system at fixed times. An increase in the concentration (c_0) of the solution ingested and a corresponding

decrease in the volume V will produce an increase in the factor $\frac{a}{V} \cdot t$,

thus an increase in the whole expression. This implies that the total remaining quantity of alcohol in the system with the same absolute amount ingested will for a certain time be greater as the concentration of the solution ingested becomes higher. By making use of formula (3) for the quantity of alcohol in the system, it has been calculated (fig. 1) what values of alcohol percentage can be expected in

the body, when 1 g alcohol pr kg body weight is ingested of solutions of 10 % (I) and 50 % (II) respectively. In addition to this a curve has been calculated with a solution of 10 % with a double value of the diffusion constant (III). Complete diffusion equilibrium between the different parts of the body has been considered to exist.

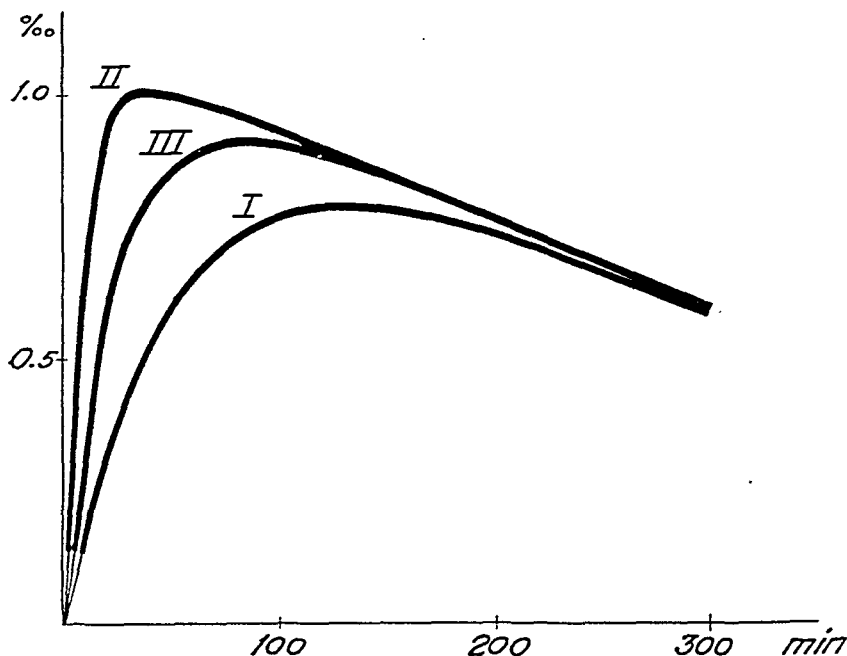


Fig. 1.

Alcohol percentage in system after ingestion of 1 g pr kg body weight.

I	Solution 10 %.	Diffusion constant 0.02
II	" 50 %	" " 0.02
III	" 10 %	" " 0.04

Rate of disappearance of alcohol from blood 0.0020 %/00 pr min.

From the curves in fig. 1 it will be seen what variations can be obtained in the size as well as in the time of the maximum of the alcohol percentage, vital factors for the appearance of intoxication symptoms. The greater the concentration of the alcohol ingested and the less the dilution taking place in the stomach owing to liquid ingested (even in the form of food), the greater will be the value for the alcohol concentration and the sooner will the maximum appear.

The influence of variations in the blood flow is seen from the following:

The blood flowing through the mucous membrane of the stomach absorbs a certain quantity of alcohol, which varies with the diffusion constant for the mucous membrane and the concentration fall between the contents of the stomach and the blood. While without running

the risk of any miscalculation it may be said that the alcohol percentage of the arteriolar blood is small as compared with that of the contents of the stomach, such an assumption is only likely for the blood after passage through the capillaries of the mucous membrane where diffusion takes place, if the flow of blood is rapid. On the other hand, if the blood flow is slow, a considerable increase in concentration must take place, and the difference in the concentration between the contents of the stomach and the blood must be decreased, which will consequently delay the diffusion.

The curve representing the rise of the alcohol percentage in the blood during its passage through the mucous membrane is also an exponential function, which asymptotically approaches the alcohol percentage of the contents of the stomach, which may be regarded as constant during the short time the same quantity of blood remains in the mucous membrane.

If c_b = blood alcohol percentage,

c_r = alcohol percentage of contents of stomach,

a = diffusion constant,

B = blood flow through mucous membrane pr time unit,

t = time ($t = 0$ means the time when the blood passes from arterioli to capillaries),

then the quantity of alcohol diffusing from the stomach to the blood may be signified according to FICK's law thus

$$\frac{dc_b}{dt} \cdot B = a (c_r - c_b)$$

which after integration gives

$$c_b = c_r - c_r \cdot e^{-\frac{a}{B} \cdot t} \quad \dots \dots \dots (4)$$

If the quantity of blood (B) flowing through the mucous membrane pr time unit is constant and passes with constant rapidity (which implies that the time for the passage of the capillaries is constant) and the alcohol percentage of the onflow of blood is minimal, the alcohol percentage in the blood of the mucous membrane (c_b) must approach a value, which stands in a constant relation to the alcohol percentage in the contents of the stomach. If this relation is defined by K and the above curve is calculated for the concentration $K \cdot c_r$, which gives the alcohol percentage of the blood of the mucous membrane after its passage through the capillaries, the following is obtained

$$K \cdot c_r = c_r - c_r \cdot e^{-\frac{a}{B} \cdot t}$$

$$K = 1 - e^{-\frac{a}{B} \cdot t} \quad \dots \dots \dots (5)$$

From this it will be seen that K increases with increased rapidity of diffusion through the mucous membrane and decreases with increased blood flow. These relations are evident from the curves (II) and (III) in fig. 2.

During the passage through the capillaries of the mucous membrane the value of the blood alcohol percentage (c_b) increases from approximately 0 to $K \cdot c_v$. The average for the values of the blood alcohol in the different parts of the capillaries can be calculated from formula (4). This calculation is shown in fig. 2, where the time for the passage

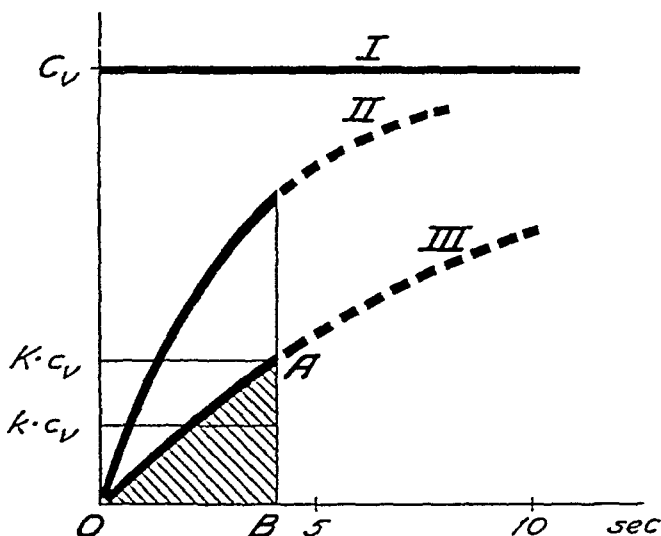


Fig. 2.

Alcohol percentage in blood during passage through capillaries of gastric mucous membrane.

- | | |
|---------------|---|
| I | Alcohol percentage in contents of stomach |
| II | " " " blood. Diffusion factor 0.3 |
| III | " " " " " " 0.1 |
| OB | Time for passage of capillaries. |
| c_v | Alcohol percentage of contents of stomach |
| $K \cdot c_v$ | " " " blood after passage through capillaries. |
| $k \cdot c_v$ | Average value of alcohol percentage of blood during passage (see text). |

through the capillaries in the mucous membrane of the stomach has been chosen to be 4 sec. The value of the alcohol percentage in the blood after the passage through the capillaries is according to the above assumption $K \cdot c_v$. The area OAB is determined by definite integration of the function (4). This area corresponds to a rectangle, the height of which represents the average of the alcohol percentage during the passage through the capillaries. This value has been called $k \cdot c_v$. If the blood flow is constant, k like K will be a constant. The relation between k and K can be calculated from formulae (4) and (5) and is

$$k = 1 + \frac{K}{\ln(1 - K)} \dots \dots \dots (6)$$

From formula (6) it is evident that k varies in a parallel manner with K .

The concentration difference determining the alcohol diffusion from the stomach to the blood — the blood alcohol percentage taken into consideration — will obviously vary between the values c_v and $c_v - K \cdot c_v$ round an average value, which according to the above can be expressed $c_v - k \cdot c_v$. The total alcohol percentage in the contents of the stomach will then, according to FICK's law, fall as follows:

$$-\frac{dc_v}{dt} \cdot V = a (c_v - k \cdot c_v) \dots \dots \dots (7)$$

Development of this gives

$$-\frac{dc_v}{c_v} = \frac{a \cdot dt}{V} (1 - k) \dots \dots \dots (8)$$

Integration

$$\ln c_v = -\frac{a \cdot t (1 - k)}{V} + y$$

$$c_v = c_{v_0} \cdot e^{-\frac{a (1 - k)}{V} \cdot t} \dots \dots \dots (9)$$

With the exception of the exponents formula (2) and formula (9) coincide completely. Taking into consideration the alcohol percentage in the blood thus makes no essential difference to the mathematical expression for the process of diffusion. The exponent of $e = -\frac{a (1 - k)}{V}$ can be empirically determined from the value of the concentration, and a constancy of the thus calculated exponent during one and the same experiment indicates that the process has been one of diffusion. On the other hand, the determination of the actual diffusion constant (a) involves considerable difficulties, since both the concentration in the blood of the mucous membrane reached during the passage and the quantity of the blood flow are not accessible for experimental determination.

Methods.

I. In one series cats have been employed after they have been kept starving for 12 hrs. The animals were anaesthetized with Pernoctone, 0.6—0.7 ml pr kg subcutaneously. In order to remove any likely remains of food and secretion the stomach was flushed several times with Ringer solution by means of a small rubber tube. Through the tube was ingested an alcoholic solution of 5—10 per cent by volume in a quantity of approximately 10 ml pr kg. Samples (0.1—0.2 ml) were taken of the contents of the stomach by means of the stomach tube at regular intervals, and after suitable dilution the alcoholic concen-

tration was analysed according to WIDMARK's micromethod (1932) with the modifications introduced by LINDE (1932). The quantitative contents of the stomach were withdrawn and measured several times during each experiment, which as a rule lasted for 1 or 2 hours.

In some cases, too, when narcosis had set in, the pylorus was ligated, and particular care was taken not to obstruct the vessels running along the pylorus, as in that case a decrease in the blood flow through the gastric wall would in all probability have ensued. After the experiments — several experiments were made on the same animal — the animal was killed and the stomach closely examined in order to control the ligation.

During the short time the experiment lasted constant circulation conditions in the mucous membrane of the stomach can be reckoned with, apart from momentary changes which took place when removing the contents quantitatively. In consequence of the inability of the stomach to absorb water (see below), a definite constancy of the volume of the alcoholic solution ingested was obtained in the experiments with ligated pylori.

II. In a second series a number of experiments were made on human subjects. 300 ml of an alcoholic solution of 5 per cent by volume were given through a thin stomach tube and samples (0.5—1.0 ml) for determination of alcohol were withdrawn every 5—10 min during 1—2 hours.

Based on the values of the alcohol concentration obtained, the factor $-\frac{a(1-k)}{V}$ of the exponent to e in formula (9) was calculated. This factor, which comprises the diffusion constant of the mucous membrane for alcohol (a), the volume of the alcoholic solution (V) and the coefficient k , which is a function of the diffusion constant (a) and the blood flow (B) — cp. formulae (5) and (6) — has been termed the *diffusion factor*. If the passage of the alcohol from the contents of the stomach to the blood is a simple diffusion process, and the factors of the exponent are constant (see p. 252) the diffusion factor will also be a constant.

In order to equalize the accidental error in the determination of the alcohol percentage, the factor has been calculated continuously between two consecutive determinations (c_1 and c_2) in one of the following ways:

a) By calculating the decrease in the alcohol percentage per min for every interval between two determinations $\left(\frac{dc}{dt}\right)$. This decrease being an expression for the rapidity of the fall in the curve, i. e. its differential coefficient, is inserted in formula (8), where c is approximated to the average between the values of the two determinations: $\frac{c_1 + c_2}{2} = c_m$.

b) By a direct insertion of the values of the determinations (c_1 and c_2) and the time interval (t) in formula (9).

For values of the diffusion factor up to 0.035, both methods give practically the same results, the difference being 0—2 %, in which case the first method (a) is considerably more simple and also has been generally used in the following experiments. When the curve falls at a greater rate, i. e. for values of the diffusion factor above 0.035, the difference will be greater. For values of 0.050—0.120 the difference between the two methods used is 2—8 %, method (a) giving the lower values, for values round about 0.170 the difference is about 25 %. This is due to the fact that with the same time intervals between the determinations there are relatively fewer ones at a more rapid fall of the curve, in consequence of which the approximation according to method (a) will be charged with a greater error.

Owing to the time intervals between two determinations generally being fairly short — 5—10 min —, the difference in concentration between two consecutive determinations will also be relatively slight. If, as is the case in the majority of the determinations, this difference amounts to approximately 20 % of the existing concentration, and the error for the alcohol determination is ± 5 % (including dilution and determination errors), the maximum error for the difference between two determinations amounts theoretically to 50 %. Practically the standard deviation (σ) lay between 14 and 35 %, the average being ± 23 %, and the standard error of the mean (ϵ) between 6 and 20 %, the average being ± 12 %.

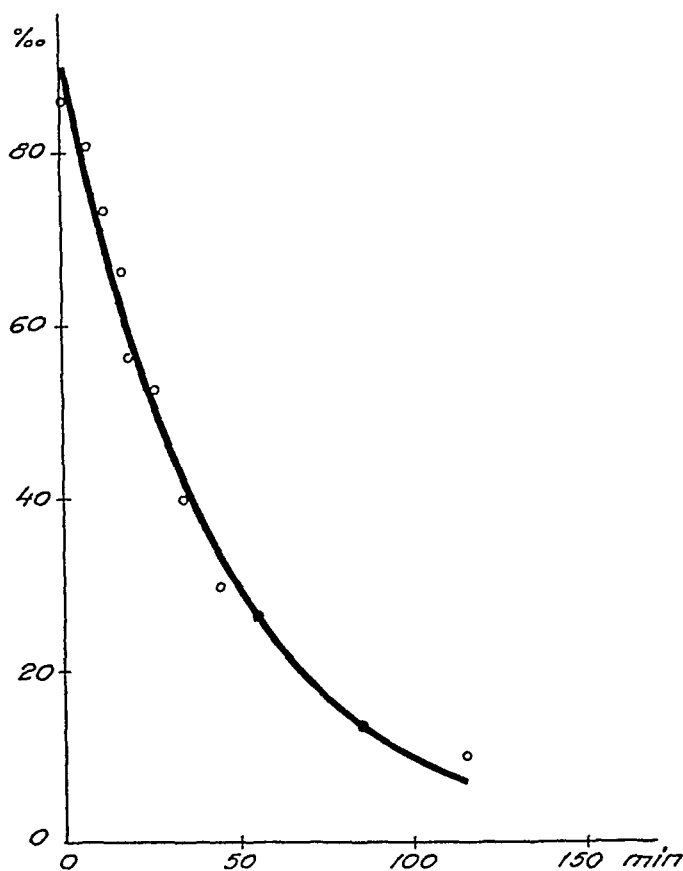


Fig. 3.

Alcohol percentage in contents of stomach.

Standard deviation ± 29 %, standard error of the mean ± 11 %.

It will be seen from fig. 3 how the determinations of the alcohol percentage in the contents of the stomach are grouped round the theoretically calculated exponential curve during the course of absorption, the standard deviation in this case being ± 29 %, the standard error of the mean ± 11 %.

Results.

A. Cats.

The following experiment on a cat may be given as a typical illustration of the course of the alcohol percentage in the con-

tents of the stomach, the pylorus being intact; the alcohol concentration was obtained through determination of samples withdrawn at regular intervals.

6. 7. — 38. Exp. 13. Cat IX. 3.6 kg. Pernoctone 0.6 ml pr kg. Pylorus intact. 30 ml of an alcoholic solution of 5 per cent by volume by stomach tube 16^h 45'. Contents of stomach, withdrawn quantitatively every 10 min, vary between 29 and 30 ml.

Time min.	Alc. conc. ‰	dc ‰	dt min.	$\frac{dc}{dt}$	c_m ‰	Diff. fact. $\frac{dc}{dt} : c_m$ method (a) I	Log c	Diff. fact. method (b) II	Difference II—I
0	29.0						1.4624		
10	22.6	6.4	10	0.64	25.8	0.0248	1.3541	0.0249	— 0.0001
20	19.5	3.1	10	0.31	21.05	0.0147	1.2900	0.0148	— 0.0001
30	15.2	4.8	10	0.48	17.85	0.0248	1.1818	0.0249	— 0.0001
40	12.6	2.6	10	0.26	13.9	0.0187	1.1004	0.0187	± 0
50	10.2	2.4	10	0.24	11.4	0.0211	1.0086	0.0212	— 0.0001
60	8.95	1.25	10	0.125	9.58	0.0181	0.9518	0.0181	± 0

Diffusion factor: average 0.020 ± 0.002

Alcohol disappearing from stomach after 30 min.: 47.5 %

, , , , , 60 min.: 69 %

From this experiment it is evident that physiologically the pylorus can remain closed as long as one hour, during which time no change seems to take place in the volume of the solution ingested. After 30 min about 48 % and after 60 min 69 % of the alcohol ingested, when given in a solution of 5 per cent by volume, had disappeared. Finally the example illustrates the constancy of the diffusion factor within the limits of error, the standard deviation being 0.0046, which is ± 23 % of the average, and the standard error of the mean being 0.002, corresponding to 10 % of the average. The difference between the methods (a) and (b) is less than a quarter of a per cent and can be neglected as long as the diffusion factor does not exceed 0.035.

Another experiment on the same animal after ligating pylorus is given below.

11. 7. 38. Exp. 14. Cat. IX. 3.6 kg. Pernoctone 0.6 ml pr kg subcut. 30 ml of an alcoholic solution of 5 per cent by volume by stomach tube 10^h 11'. Pylorus ligated. Contents of stomach, withdrawn quantitatively every 10 min, vary between 29 and 30 ml.

Time min.	Alc. conc. ‰	dc ‰	dt min.	dc dt	cm ‰	Diff. fact. method(a) I	Log c	Diff. fact. method(b) II	Difference II—I
0	27.6						1.4409		
5	24.7	2.9	5	0.58	26.15	0.0222	1.3927	0.0222	± 0
10	22.0	2.7	5	0.54	23.35	0.0231	1.3424	0.0232	- 0.0001
20	18.6	3.4	10	0.34	20.3	0.0168	1.2695	0.0168	± 0
30	14.9	3.7	10	0.37	16.75	0.0221	1.1732	0.0222	- 0.0001
40	12.1	2.8	10	0.28	13.5	0.0207	1.0828	0.0208	± 0
50	10.1	2.0	10	0.20	11.1	0.0180	1.0043	0.0181	- 0.0001
75	6.8	3.3	25	0.13	8.45	0.0154	0.8325	0.0158	- 0.0004

Diffusion factor: average 0.020 ± 0.001

Alcohol absorbed from stomach after 30 min.: 46 %

60 min.: 69 %

These two experiments, performed on the same animal, fully agree (exp. 13 and 14). Here 46 % of the alcohol ingested disappeared from the stomach after 30 min and 69 % after 60 min. As the volume of the solution had been constant during the whole time — water not being absorbed from the stomach — the disappearance of alcohol must be attributed to an absorption. The diffusion constant had the same value as with intact pylorus: 0.020 ± 0.001 , the standard deviation being relatively smaller: ± 0.0028 , that is 14 % of the average, and the standard error of the mean ± 0.001 , which is 5 % of the average. No essential difference could be seen between methods (a) and (b) when calculating the diffusion factor, and in the following the diffusion factor will be calculated according to method (a).

The ingestion of alcohol in a solution of 16—17 % shows similar conditions, as is illustrated by the following example.

27. 8. 38. Exp. 19. Cat XI. 2.91 kg. Pernoctone 0.7 ml pr kg subcut. 30 ml of an alcoholic solution of 16.7 per cent by volume by stomach tube 12^h 30'. Pylorus ligated.

Time min.	Alc. conc. ‰	dc ‰	dt min.	$\frac{dc}{dt}$	c_m ‰	Diff. fact.
0	86.3					
5	80.8	5.5	5	1.1	83.6	0.013
10	73.0	7.8	5	1.56	76.9	0.020
15	66.0	7.0	5	1.4	69.5	0.020
20	56.7	9.3	5	1.86	61.4	0.030
25	52.5	4.2	5	0.84	54.6	0.015
35	40.1	12.4	10	1.24	46.3	0.027
45	30.0	10.1	10	1.01	35.1	0.029
55	26.2	3.8	10	0.38	28.1	0.014
85	13.4	12.8	30	0.43	20.0	0.022
115	10.0	3.4	30	0.11	11.7	0.010

Diffusion factor: average 0.020 ± 0.002

Alcohol absorbed from stomach after 30 min.: 46 %

„ „ „ „ 60 min.: 72 %

„ „ „ „ 90 min.: 93 %

The diffusion factor is in this experiment of the same magnitude as when alcohol in a solution of 5 per cent by volume is given, which also supports the assumption that absorption is a diffusion process. As in some other cases, another fact appears in this example. The diffusion factor, which has on the whole been constant, — 0.021 ± 0.002 — shows a certain tendency to decrease towards the end of the experiment and its last value is as low as 0.010, a figure which lies outside the normal limit of variation. The most likely reason for this is a deterioration of the blood flow in the mucous membrane, i. e. a decrease of B [see formulae (4) and (6)] and consequently in the whole of the diffusion factor, or else a decrease in the mobility of the gastric wall with incomplete mixing.

When the stomach is emptied during the experiment, the alcohol concentration in the contents of the stomach takes another course. A typical example of this in a case of intact

pylorus, where a more or less continuous decrease in the volume of the solution ingested has taken place, is illustrated by exp. 6.

9. 6. 38. Exp. 6. Cat VII. 4.0 kg. Pernoctone 0.7 ml pr kg subcut. 42 ml of an alcoholic solution of 4.8 per cent by volume by stomach tube 9^h 55'. Pylorus intact.

Time min.	Volume of contents of stomach ml	Alc. conc. ‰	dc ‰	dt min.	c _m ‰	Diff. fact.
0	41	31.5				
5	37	27.7	3.8	5	29.6	0.026
15	31	20.3	7.4	10	24.0	0.031
25	14	14.9	5.4	10	17.6	0.031
35	18.5	9.8	5.1	10	12.4	0.041
50	14.5	6.46	3.35	15	8.1	0.028
65	15	3.4	3.05	15	4.9	0.041
80	10.5	2.3	1.1	15	2.85	0.026

Diffusion factor: average 0.032 ± 0.003

Alcohol disappearing after 30 min.: 61 %

„ „ 60 min.: 86 %

In this case the diffusion factor was 0.032 ± 0.003 ; after 30 min 61 %, after 60 min 86 % of the alcohol ingested had disappeared. Another experiment on the same animal — also with an intact pylorus — gave a diffusion factor of 0.039 ± 0.011 ; 77 % of the alcohol ingested had disappeared after 30 min (exp. 7, table 1). These values are to be compared with the results of experiments on the same animal after ligating pylorus (exp. 8 and 9, table 2). After ligation the diffusion factor was 0.017 and 0.021 respectively, as against 0.032 and 0.039 before; after 30 min an average of 38 % of the alcohol ingested had been absorbed against an average of 69 % when the pylorus was left intact. The diffusion factor had thus increased with 84 %, corresponding to an increase in the amount of alcohol absorbed after 30 min of 82 %. The increase of the diffusion factor in the case of non-ligated pylorus will thus be caused by the decrease of the volume (V) of the solution ingested, which fully agrees

with the formulae adopted [cp. formula (9), where V is to be found in the denominator of the diffusion factor]. The difference between the diffusion factors in these experiments before and after ligating pylorus is statistically significant. — When no change in the volume of the solution ingested takes place, the diffusion factor becomes constant, as does also the amount of alcohol absorbed (cp. exp. 12 and 13, table 1 and exp. 14 and 15, table 2).

A survey of the experiments with intact pylori is given in table 1. The strength of the alcoholic solution was 5 per cent by volume, c:a 10 ml solution was given pr kg body weight.

Table 1.

Survey of experiments with intact pylori on cat.

Cat nr	Exp. nr	Alc. conc. Per cent by vol- ume	Per cent alcohol absorbed after			Diffusion factor	Volume of contents of stomach ml
			30'	60'	120'		
III	2	5	32.5	45	68.5	0.011 ± 0.001	Constant
IV	4	5	38.5	58	—	0.015 ± 0.003	Decreasing
V	5	5	42	63.6	—	0.019 ± 0.004	'
VII	6	5	61	86	—	0.032 ± 0.003	'
	7	5	77	—	—	0.039 ± 0.011	'
VIII	10	5	33	—	—	0.014 ± 0.002	Constant
	11	5	37	—	—	0.016 ± 0.001	Decreasing
IX	12	5	61.5	76	—	0.024 ± 0.004	Constant
	13	5	47.5	69	—	0.020 ± 0.002	'

Diffusion factor with intact pylorus: average $0,021 \pm 0,003$ (9 exp.)

Alcohol disappearing after 30 min.: 48 % (6 exp.)

60 min.: 67 %

The average of the diffusion factors, the volume of the solution ingested being constant, was 0.017 ± 0.003 , the average with decreasing volume 0.024 ± 0.005 , the difference not being significant as the number of experiments was too small to allow of statistics.

Below is given a similar survey of cases with ligated pylori (table 2).

Table 2.

Survey of experiments with ligated pylori on cat.

Cat nr	Exp. nr	Alc. conc. Per cent by vol- ume	Per cent alcohol absorbed after			Diffusion factor	Volume of contents of stomach ml
			30'	60'	120'		
III	3	5	16.5	41.5	74.5	0.007 ± 0.002	Constant
VII	8	5	31	54	78	0.017 ± 0.002	,
	9	5	44	72	—	0.021 ± 0.002	,
IX	14	5	46	69	—	0.020 ± 0.001	,
	15	5	52	75	—	0.023 ± 0.002	,
X	16	5	50	—	—	0.018 ± 0.003	,
	17	5	42.5	—	—	0.019 ± 0.002	,
XI	18	5	43	—	—	0.019 ± 0.003	,
	19	16.7	46	72	93	0.021 ± 0.002	,

Diffusion factor with ligated pylori (alc. 5 %): average 0.018 ± 0.002 (8 exp.)

Alcohol absorbed after 30 min.: 38 % (5 exp.)

, , , 60 min.: 62 % ,

In the first series of experiments — with intact pylori — a greater percentage of the alcohol had disappeared from the stomach after a certain time than compared with the corresponding values in the series with ligated pylori. The difference will probably be due to a partial emptying of the contents of the stomach into the intestines — as indicated by the decreasing values of the volume V — rather than to a more rapid absorption in the cases of intact pylori, for in the experiments, where the volume of the solution ingested had been constant throughout the experiment, the magnitude of the diffusion factors, in average 0.017 ± 0.003 , was the same as in the cases with ligated pylori: 0.018 ± 0.002 . Whether the alcohol after passage into the intestines was absorbed more quickly or slowly cannot be determined by this method.

The low values of the diffusion factors in the first experiments in the two series (exp. 2 and 3) as compared with the average, will most likely be due to inferior technique and also perhaps to inferior circulation conditions in the stomach, which may cause a lower diffusion factor.

B. Human Subjects.

I. Normal Individuals.

As an extension of the results obtained on cat, a series of experiments have been carried out on human subjects in order to see to what extent the above laws may be applied here.

The example below may serve to illustrate a normal experiment.

23. 5. 38. Exp. 10. H. J. 67 kg. 300 ml of an alc. sol. of 5 % by vol. by stom. tube.

Time min.	Alc. conc. ‰	dc ‰	dt min.	cm ‰	Diff. fact.
0	32.6				
5	29.6	3.0	5	31.1	0.019
10	28.2	1.4	5	28.9	0.010
15	25.8	2.4	5	27.0	0.018
20	24.0	1.8	5	24.9	0.015
25	21.9	2.1	5	23.0	0.018
30	19.2	2.7	5	20.6	0.026
35	17.1	2.1	5	18.2	0.023
40	15.0	2.1	5	16.1	0.026
45	13.5	1.5	5	14.2	0.021
55	10.6	2.9	10	12.1	0.024
65	7.9	2.7	10	9.3	0.032

Diffusion factor, before emptying: average 0.016 ± 0.002

, , after , , 0.025 ± 0.002

Alcohol absorbed after 30 min: 41 %

, , , 60 : 72 %

As will be seen the diffusion factor shows good constancy up to 30 min — 0.016 ± 0.002 — then to rise to 0.026 and afterwards to assume a constant value but now on a somewhat higher level: 0.025 ± 0.002 , the difference being statistically significant. Based on the experiments on animals with intact and ligated pylori, it may be assumed that the reason for the sudden change in the diffusion factor is a partial emptying of the stomach with a subsequent decrease in the diffusion factor [see formula (9)]. After this the volume seems to keep constant, for the diffusion factor likewise remains constant.

An experiment was also made on the same human subject with a somewhat stronger alcoholic solution in the form of beer with an addition of extra alcohol, corresponding to a solution of 8 per cent by volume. This is illustrated by fig. 4.

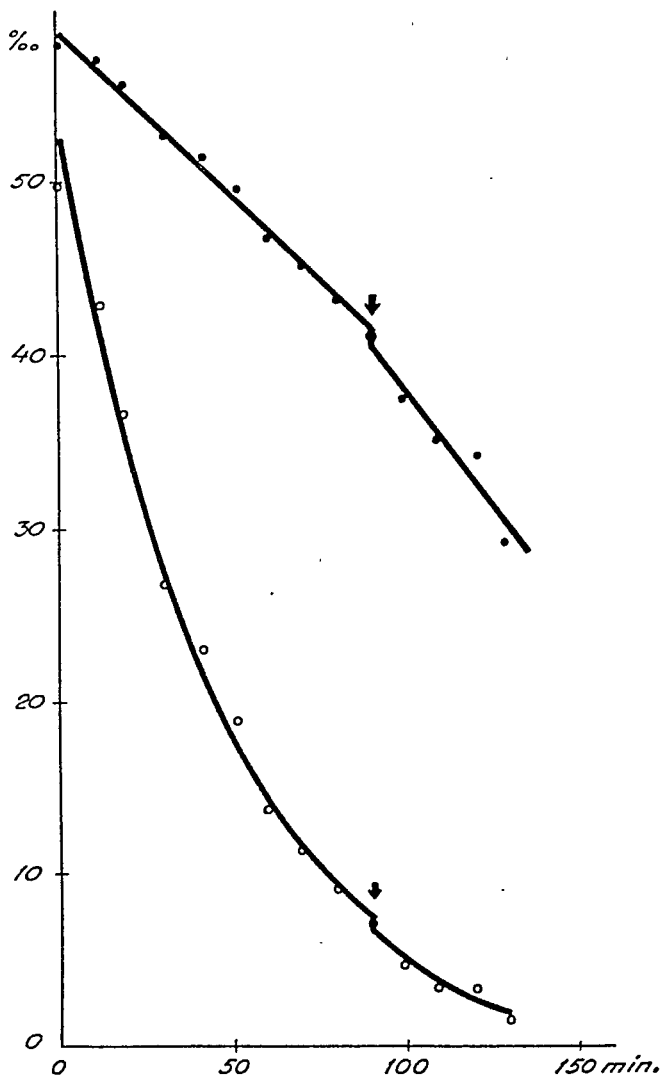


Fig. 4.

Alcohol percentage in contents of stomach.

Exp. 11. J. H. 67 kg. 330 ml beer + 15 ml alc. abs., corresponding to a solution of 8 per cent by volume, by stomach tube 8^h.

○ — ○ — ○ alcohol percentage in contents of stomach

● — ● — ● logarithms for alcohol percentage

↓ emptying of stomach at 90 min.

Diffusion constant before emptying: 0.021 ± 0.002

„ „ after „ : 0.032 ± 0.008

This experiment shows the agreement between the results at repeated experiments even on human material. The diffusion factor remains constant: 0.021 ± 0.002 , for as long as 90 min, then suddenly to rise to 0.034 and afterwards to remain more or less constant with an average of 0.032 ± 0.008 . As in exp. 10 the reason for this sudden change must be ascribed to a partial emptying of the stomach with a corresponding decrease in the volume of the solution ingested — about 33 % when calculated from the change in the diffusion factor [formula (9)]; afterwards a constancy of the volume again appears, as indicated by the constancy of the diffusion factor. — The sudden change in the course of absorption which takes place after 90 min, is not clearly visible when forming a curve of the absolute values representing the alcohol percentage of the contents of the stomach, but only after calculating the diffusion factors (as in exp. 10) or by forming the theoretical exponential curve by means of the method of the least squares (curve $\circ - \circ$ in fig. 4) or by plotting the logarithmic values of the alcohol percentage ($\bullet - \bullet - \bullet$ in fig. 4).

The following example where the diffusion factor increases gradually, would then be connected with a continuous emptying of the stomach.

3. 6. 38. Exp. 15. S. M. B. 80 kg. 300 ml of an alc. sol. of 5 % by vol. through stomach tube 8^h.

Time min.	Alc. conc. ‰	dc ‰	dt min.	c _m ‰	Diffusion factor
0	31.7				
5	27.2	4.5	5	29.5	0.030
16	13.6	18.6	11	20.4	0.061
20	7.75	5.85	4	10.7	0.136
30	0				

Alcohol disappearing after 30 min: 100 %.

A survey of the normal cases is given in table 3. In certain cases the values of the diffusion factors during the experiment have shown a sudden increase. In analogy to what has already been said this increase will be due to a partial emptying of the

stomach, and the time for this, calculated from the change in the diffusion factor, is also to be seen from table 3.

Table 3.

Survey of experiments with alcohol test meal on normal individuals.

Subj.	Exp. nr	Alc. conc. Per cent by volyme	Per cent alc. dis- appearing from stom- ach after			Stomach emptied after	Diffusion factor		
			30'	60'	120'		before after emptying		Differ- ence II—I
							I	II	
D	5	5	88	—		10'	0.029	0.090	0.061
	12	5	70	96		25'	0.037	0.060	0.023
M	8	5	58	85		—	0.033	—	—
J	10	5	41	72		25'	0.016	0.025	0.009
	11	8	46	73	96	90'	0.021	0.032	0.011
B	15	5	100	—		5'	0.030	0.098	0.068
W	16	5	45	78		39'	0.019	0.049	0.030

Diffusion factor before emptying: average 0.025 ± 0.003 (6 exp.)

„ „ after „ „ 0.059 ± 0.013 „

Alcohol disappearing after 30 min: 52 % (5 exp.)

„ „ „ 60 min: 81 % „

From table 3 it is evident that the diffusion factors in the different individuals previous to the supposed emptying of the stomach are of approximately the same magnitudes with an average of 0.025 ± 0.003 ; after emptying, however, the values rise considerably: in average to 0.059 ± 0.013 . The average of the increase of the diffusion factors after emptying, when calculated from 6 experiments, is 0.034 ± 0.010 , which is statistically significant.

II. Patients.

Some experiments have been made on patients, especially those suffering from achlorhydria and gastritis, with an attempt to apply the above experiences on clinical material. The alcohol was

given as a test meal: 300 ml of an alcoholic solution of 5 per cent by volume through stomach tube, and samples were withdrawn every 5—10 min. The cases were kindly placed at our disposal by doc. G. NYLIN, Serafimerlas., med. clin. II. A survey of the cases is given in table 4.

Table 4.

Survey of experiments with alcohol test meal on patients.

Subj.	Exp. nr	Alc. conc. Per cent by volume	Per cent alc. disappearing from stomach after		Stomach emptied after	Diffusion factor		
			30'	60'		before emptying	after emptying	Difference II—I
						I	II	
Lg	1	5	72	96	40'	0.036	0.077	0.041
Lq	2	5	60	92	30' and 40'	0.030	0.087	0.057
K	6	5	87	—	—	—	0.050	
S	9	5	70	91	25'	0.036	—	
Ja	13	5	87	—	25'	0.043	0.082	0.039
G	14	5	46	88	47'	0.018	0.075	0.057

Diffusion factor before emptying: average 0.032 ± 0.006 (4 exp.)

„ „ after „ „ 0.080 ± 0.003 „

Alcohol disappearing after 30 min: 62 % (4 exp.)

„ „ „ 60 min: 92 % „

According to table 4, there seems to be no essential deviation from the results found on normal individuals. The figures show a certain tendency to higher diffusion factors among the clinical material, which may possibly be due to a more rapid disappearance of the alcohol from the stomach. There may be two reasons for this, firstly a more rapid absorption, something not altogether unusual in the case of gastritis, secondly a more rapid disappearance owing to the stomach emptying more rapidly, this being a well known fact in the case of achlorhydria. The material is however insufficient to allow of statistics, and should be completed with absolute figures of the volume of the contents of the stomach if it is to be a guide as to which of the possibilities predominates.

Conclusions.

By continuously following the variations in the alcohol percentage of an alcoholic solution ingested in the stomach, it has been possible to obtain the curve for the disappearance of alcohol from the stomach. In cases where the pylorus has been ligated or repeated measurements have proved convincing that no change has taken place in the volume of the solution ingested, the disappearance of alcohol must be ascribed to absorption. Assuming that absorption follows the laws of diffusion and taking into consideration the volume and concentration of the solution ingested, the percentage of blood alcohol and the blood flow through the mucous membrane of the stomach, it has been possible to obtain a so-called *diffusion factor* which, provided the volume ingested and the blood flow are constant, will likewise be a constant. Although there are some variations in the size of the diffusion factor, a survey of all the cases shows its constancy and thereby the tenability of the assumption, to which the experiments have been adapted, *i. e.* that the passage of ethyl alcohol from the gastro-intestinal tract to the blood is a simple process of diffusion. The deviations from the constancy of the diffusion factor can equally be explained by the changes of the other elements¹ forming this factor. The formulae set up have also proved applicable on human subjects.

Summary.

After a survey of earlier literature on the passage of ethyl alcohol from the gastro-intestinal tract to the blood, the factors are discussed that may possibly have to bear on this passage, the basis of the discussion being the laws of diffusion. From these laws formulae have been deduced, in which the percentage of the blood alcohol, the blood flow through the mucous membrane of the stomach and the concentration and the volume of the alcoholic solution ingested have been taken into consideration. The validity of these formulae is not only confined to the diffusion of alcohol but also applies to all the cases in which a pure process of diffusion is concerned.

By means of a series of determinations of the alcohol percentage in the contents of the stomach of cat with intact and ligated

pylori as well as on human subjects — both normal individuals and patients — by means of fractional withdrawal of ingested alcohol, an attempt has been made to show that the passage of alcohol through the mucous membrane of the stomach is to be regarded as a pure process of diffusion. The results of the experiments are:

1) The absorption of ethyl alcohol from the stomach follows the laws of diffusion. This means that the greater the concentration of the alcohol ingested, the more rapidly will absorption take place with a higher and earlier blood alcohol maximum (cp fig. 1). The so-called diffusion factor $\frac{a(1-k)}{V}$ is constant in the case of constant blood flow and constant volume.

2) A cat with ligated pylorus absorbs in 30 min about 38 % of the alcohol ingested, when given in a solution of 5 per cent by volume, about 62 % after 60 min and 76 % after 120 min; of a solution of 17 per cent proportionally more is absorbed.

3) On human subjects with closed pylori the corresponding figures for a solution of 5 per cent by volume are 40 % after 30 min, 70 % after 60 min and about 100 % after 120 min. If the stomach is emptied, on the other hand, the whole quantity of alcohol ingested can disappear in less than 30 min.

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Evidence for two Phases in the Regeneration of Visual Purple.¹

By

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(With 5 figures in the text.)

It was noted by ZEVI (1939) that, although the process of regeneration of visual purple in live frogs had a high temperature coefficient, this process in excised opened eyes could not be accelerated by a rise in temperature. Regeneration in isolated eyes was also relatively slow, and, in order to obtain an equally slow rate of regeneration in the live animal, the temperature had to be brought down to about 8°. The isolated eyes thus behaved as if they had been lacking that phase of regeneration which in the intact frog is particularly sensitive to temperature.

Some observations on the effect of pilocarpine and atropine on V. P. (visual purple) regeneration are of interest in this connexion and will therefore be reported. They were made in order to test with a quantitative technique some old observations by AYRES and KUEHNE (1882) on rabbits and by DRESER (1886) on frogs. Pilocarpine was used by them on account of its favourable effect on secretion and atropine as the corresponding inhibitory substance. This type of problem and the general inferences to be drawn from experiments of this kind do not appear to us in the same light to-day as in 1880. Nevertheless the results seem to justify a repetition of the experiments with the improved technique now at our disposal. The old authors reported that atropine had no effect on the regeneration of visual purple but that pilocarpine enhanced it. These observations were confirmed by AMENOMIYA (1930).

¹ Received 17 September 1940.

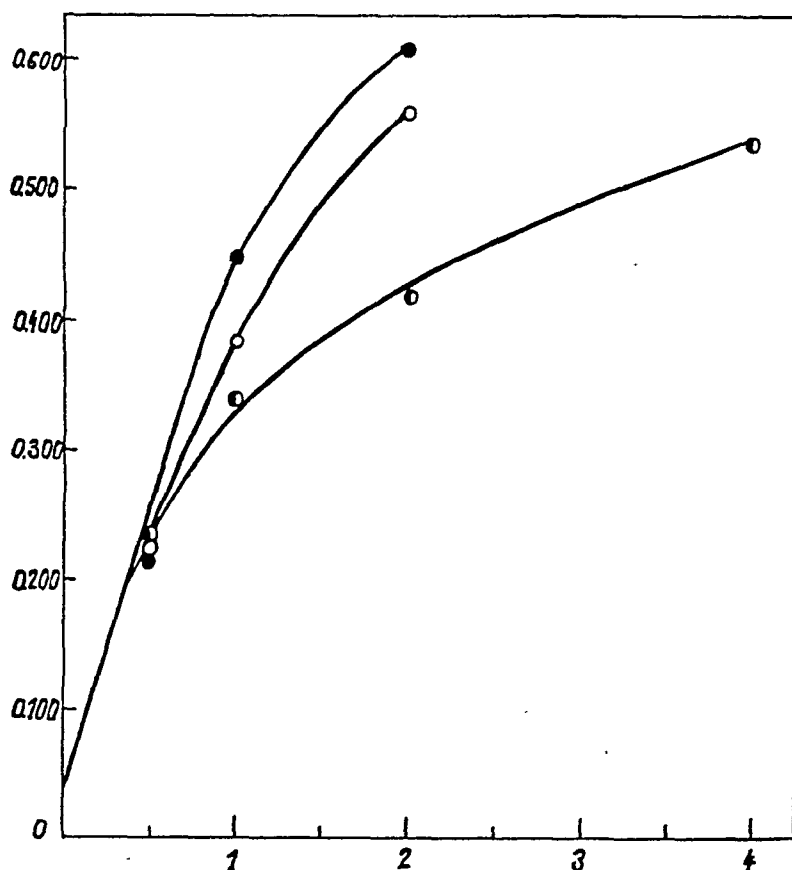


Fig. 1. Visual purple regeneration in live frogs at 22.4°C: ● after injection of 5 mg pilocarpine (23 eyes). ○ after injection of 5 mg atropine (28 eyes). ○ controls (22 eyes). Abscissae: time in the dark in hrs. Ordinates: density of visual purple. Later figg. similarly marked.

Technique.

As experimental animals served Hungarian frogs. The visual purple was extracted with 2 per cent digitonin (TANSLEY, 1931). Each retina was separately extracted with 1 ccm of the solvent and left in it for 40 min. at room temperature. Then followed centrifugation for 20 min. at 3,800 rev./min. Of the clear extract 0.4 ccm was measured into an absorption trough of 20 mm length. This was put into the beam of a light of wave-length 0.498μ and its absorption determined with the aid of a photocell and an electrometer. For details, see GRANIT, HOLMBERG and ZEVI (1938) and ZEVI (1939).

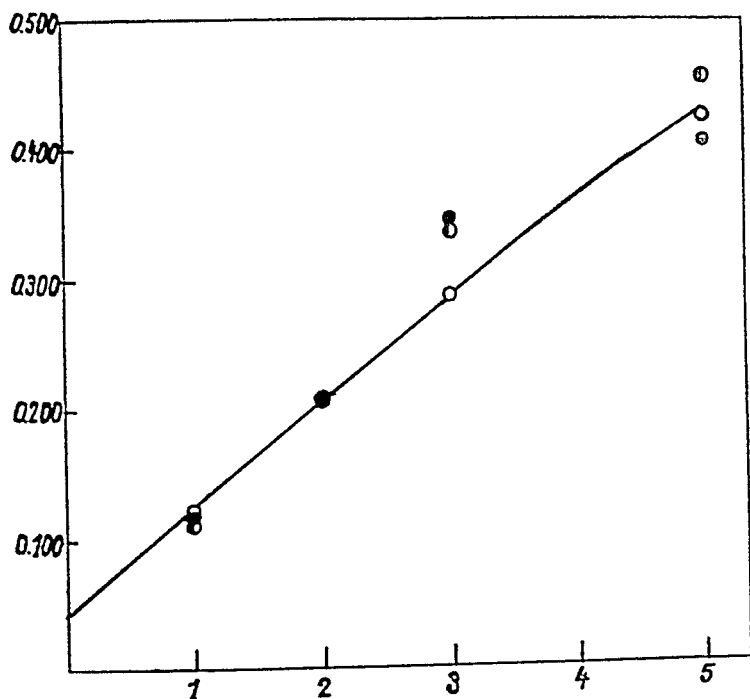


Fig. 2. Visual purple regeneration in live frogs at 8.0°: ● after injection of 5 mg pilocarpine (31 eyes). ◐ after injection of 5 mg atropine (29 eyes). ○ controls (21 eyes).

Visual purple concentration will be expressed in the usual manner as the difference in density (which is $\log \frac{\text{light incident}}{\text{light transmitted}}$) between the original unbleached extract and the fully bleached extract.

Results.

1. Regeneration in live frogs.

Frogs dark adapted overnight were light adapted for one hour to 20,000 m. c. Immediately after light adaptation some animals received 5 mg. pilocarpine into the lymph sac, others the same amount of atropine, and some served as controls. The frogs were then left to dark adapt in a water bath at constant temperature (ZEWI, 1939). They were removed from the water bath after suitable intervals in the dark, decapitated in red light, after which the eyes were analyzed for visual purple. In this manner regeneration curves were obtained for two temperatures, 22.4 and 8° C.

Such curves are shown in figg. 1 and 2. They are not drawn from origo but from a point corresponding to a V. P. concentration of 0.040, which is the average minimum to which visual purple can be brought by light adaptation (ZEWI, 1939). The readings marked in the figures are averages of several eyes.

Fig. 1 shows that regeneration in all cases initially follows a common course but that later on pilocarpine has slightly accelerated the rate of formation of visual purple. Atropine very definitely slows down regeneration.

Fig. 2 gives the additional significant information that the slow rate of regeneration at 8° is uninfluenced by either drug.

2. Regeneration in opened isolated eyes of animals injected with pilocarpine or atropine.

The dark adapted frogs received an injection of 5 mg. pilocarpine or atropine and were then light adapted for one hour to 20,000 m. c. After this the eyes were removed, opened, and left to dark adapt at 22.2°.

Fig. 3 shows that pilocarpine had no effect whatsoever, but that, contrary to expectation, atropine now very definitely improved the rate of V. P. regeneration.

3. Regeneration in opened isolated eyes treated with pilocarpine or atropine.

The eyes of dark adapted frogs were removed and opened in red light and thereafter illuminated for 30 min. with a 1,000 W. lamp at a distance of 40 cm. After light adaptation in this manner the right eye received one drop of a 1 per cent solution of atropine or pilocarpine. The left eye served as control and had a drop of Ringer. Then followed dark adaptation at 22.4° and the amount of visual purple was determined for each pair of eyes in parallel. This procedure is based on the fact that V. P. concentration in right and left eye does not as a rule differ by more than, on an average, 1 per cent, provided that a sufficient number of eyes be used (GRANIT ET AL. 1938, ZEWI 1939).

Fig. 4 shows that regeneration was suppressed after pilocarpine. The less definite result of fig. 5 illustrates that atropine suppresses regeneration.

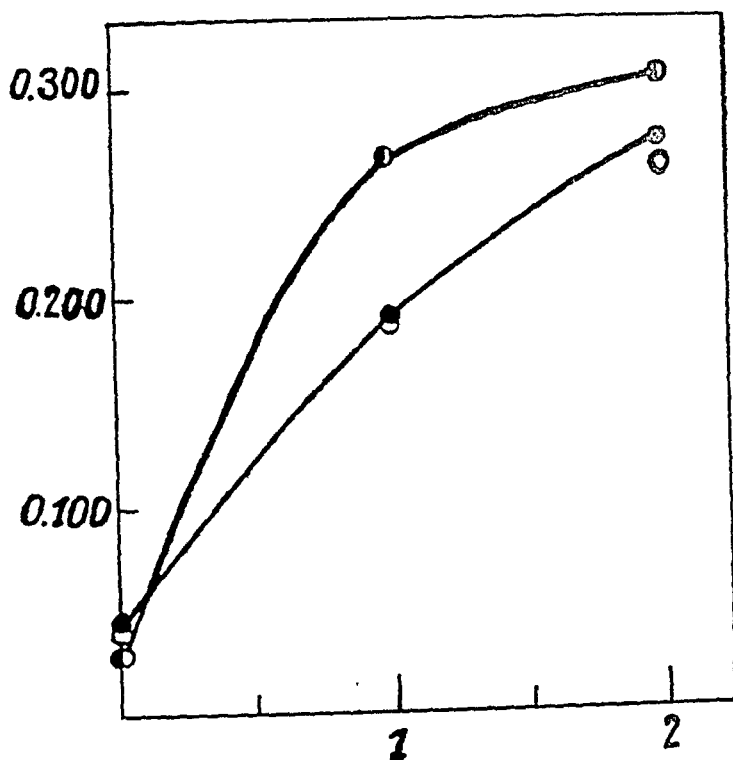


Fig. 3. The regeneration of visual purple in excised opened eyes at 22.2°. Before light adaptation the frogs received an injection of 5 mg of ● pilocarpine or ○ atropine into the lymph sac. Controls are marked ○. 12, 14, and 15 eyes were used for the respective series.

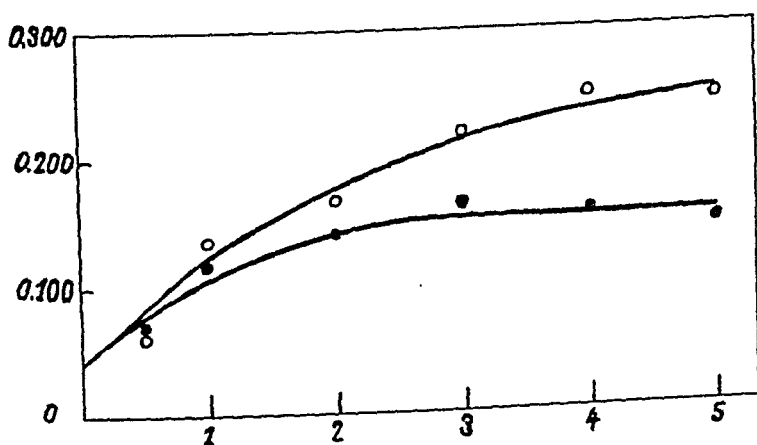


Fig. 4. Visual purple regeneration in 18 excised and opened eyes at 22.4° treated with pilocarpine ● compared with 18 controls ○, which received only a drop of Ringer.

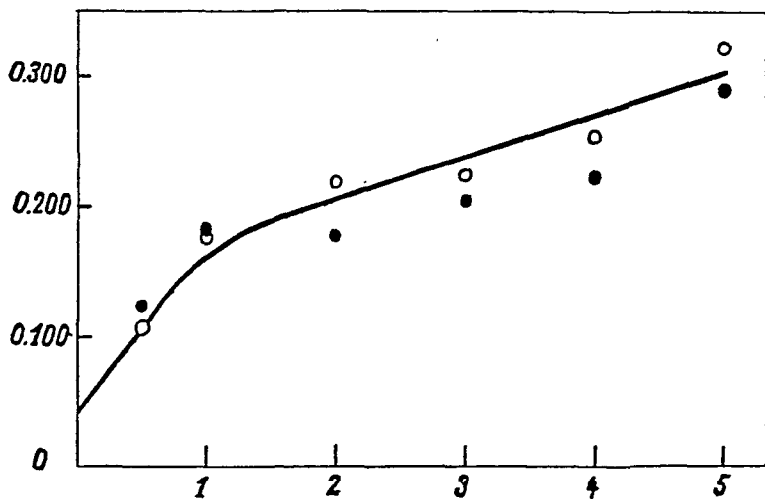


Fig. 5. Visual purple regeneration in 18 excised and opened eyes at 22.4° treated with atropine ●, compared with 18 controls O, which received only a drop of Ringer.

Discussion.

The interesting results are those of figg. 1 and 2 in which a differentiation of the regenerative component processes was carried out by means of temperature. At 8° neither drug had any influence but at 22.4° pilocarpine somewhat accelerated and atropine definitely slowed down the rate of regeneration of visual purple. This must be regarded as further evidence in favour of the dual nature of V. P. regeneration. Only the component sensitive to temperature is influenced by the drugs. It is hardly probable, despite the low temperature used, that within the 5 hours during which the process was followed, absorption of the drugs from the lymph sac should not have taken place.

Very curious is the fact, shown in fig. 3, that excision of the eye of the atropinized frog leads to an improvement in the rate of regeneration of visual purple as compared with *control eyes*. The same improvement did not appear in the live frog at 8° . It reminds one of observations by JÄRVI (1935) according to which the secretory cells of trachea and larynx of cats and guinea pigs after atropinization are richly filled with secretory substance although secretion outwards cannot take place. Atropine may favour some preparatory process, say, in the retinal pigment cells but this favourable effect cannot lead to increased formation of visual

purple before the eye is removed and thereby withdrawn from the direct influence of the drug.

Very little use can be made of the circumstance that both drugs impede regeneration of visual purple when dropped into excised opened eyes. This may interfere directly with the state of the tissues which for good regeneration must be normal (ZEWI, 1939).

I am indebted to Professor RAGNAR GRANIT, Stockholm, for technical support and criticism of this work as well as for its translation into English.

Summary.

The concentration of visual purple has been determined photo-electrically during dark adaptation of previously light adapted frogs at 22.4 and 8°.

At the higher temperature pilocarpine slightly enhances, and atropine definitely suppresses the rate of regeneration of visual purple in live frogs. At the lower temperature neither substance has any effect on the regenerative processes.

The results are interpreted as further evidence in favour of the dual nature of visual purple regeneration. Only the process sensitive to temperature is in this particular instance sensitive to the drugs.

Some results with regeneration in excised eyes under the influence of pilocarpine and atropine are also reported.

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Eine einfache und für klinische Zwecke geeignete Mikromethode zur Bestimmung des Harnstoffstickstoffs (UrN^+) im Blute durch Ureasebehandlung und direkte Nesslerisierung.¹

Von

WILHELM OHLSSON.

KAY und REID (1934) haben nachgewiesen, dass der optimale pH-Wert für die Urease zwischen 6.6 und 7.0 liegt, und aus ihren Versuchen geht hervor, dass noch bei einem p_H von 6.2 fast optimale Enzymwirkung erzielt wird. Bei den bisherigen Methoden zur Bestimmung des Harnstoffs mit Hilfe von Urease hat es sich im allgemeinen denn auch als notwendig erwiesen, eine besondere Pufferlösung zu verwenden, um einen geeigneten pH-Wert zu erhalten.

Bei der von mir schon früher beschriebenen Methode zur Bestimmung des Reststickstoffs (1937) wird die entnommene Blutprobe in eine sog. Molybdatlösung gebracht, die Natriummolybdat und Kaliumsulfat enthält. Diese Molybdatlösung, deren eigentliche Aufgabe es ist, als eiweissfällende Lösung zu dienen, hat ein p_H von 6.2—6.5 und erwies sich als ein ausgezeichnetes Medium für die Ureasewirkung. Die aufgefangene Blutprobe kan deshalb direkt mit Urease versetzt werden, und das besondere Zusetzen einer Pufferlösung erübrigt sich.

Das Prinzip der Methode besteht darin, dass der Harnstoff des Blutes mit Hilfe der Urease in Ammoniumkarbonat übergeführt wird, dessen Stickstoffmenge, nach Fällen des Eiweisses, mit dem Nessler'schen Reagens direkt kolorimetrisch bestimmt werden kann.

¹ Der Redaktion am 28. September 1940 zugegangen.

Die Mehrzahl der für diese Methode erforderlichen Lösungen stimmt mit den bei der oben erwähnten Methode zur Reststickstoffbestimmung zur Verwendung kommenden überein. Es muss nur noch eine Urease-Lösung und eine Gummi-arabicum-Lösung bereitet werden.

Erforderliche Lösungen.

Molybdatlösung: 10 ml 10 %ige Natriummolybdatlösung (pro analysi) und 6.5 g Kaliumsulfat (pro analysi) werden in einen 500 ml fassenden Messkolben gebracht und bis zur Marke mit destilliertem Wasser verdünnt.

Fällungslösung: 40 ml 1-normaler Schwefelsäure werden in einen 300 ml fassenden Messkolben gebracht und bis zur Marke mit destilliertem Wasser verdünnt.

Standardlösungen: 0.4716 g getrocknetes Ammoniumsulfat (pro analysi) werden im Messkolben in destilliertem Wasser bis auf die Menge von 1 Liter gelöst. Die Lösung enthält 0.1 mg Stickstoff pro ml.

Standard I: 20 ml der obigen Standardstammlösung werden auf 200 ml verdünnt. Enthält 0.01 mg Stickstoff pro ml.

Standard II: 80 ml der obigen Standardstammlösung werden auf 200 ml verdünnt. Enthält 0.04 mg Stickstoff pro ml.

Stammlösung für das Nessler'sche Reagens: In einem Jenakolben, der 500 ml Wasser von 70° enthält, werden 90 g Jodkalium und 100 g Quecksilberjodid gelöst. Die Lösung wird abgekühlt, in einen 1 Liter fassenden Messkolben gebracht und bis zur Marke verdünnt.

Nessler'sches Reagens: 450 ml der oben erwähnten Nessler'schen Stammlösung werden mit 2 100 ml 10 %iger Natronlauge und 1 200 ml destilliertem Wasser versetzt, worauf sorgfältig gemischt wird. Die Lösung wird in dunkler Flasche aufbewahrt.

Sämtliche oben beschriebenen Lösungen sind dieselben, die bei der oben erwähnten Methode zur Reststickstoffbestimmung zur Verwendung kommen.

Ureaselösung: 1 Tablette Urease Squibb (E. R. Squibb & Son, New York) zu 0.1 g wird zerdrückt und in 1 ml destilliertem Wasser aufgeschwemmt. Diese Lösung wird bei Vornahme der Bestimmung jedesmal frisch bereitet.

Gummi-arabicum-Lösung: 1 %ig.

Ausführung.

Behandlung der Blutprobe: In ein Zentrifugenglas, das 8 ml Molybdatlösung enthält, werden 0.2 ml Blut gebracht, wobei die Blutpipette durchgespült wird. Es werden 2 Tropfen Ureaselösung hinzugegeben. Sorgfältiges Mischen durch vorsichtiges Schütteln. Stehenlassen der Mischung 30 Minuten lang bei Zimmertemperatur. Darauf werden 2 ml der Fällungslösung hinzugefügt und nach Mischen, 10 Minuten lang kräftig zentrifugiert. Das klare Zentrifugat wird dekantiert, z. B. in ein anderes Zentrifugenglas, und 8 ml davon werden in ein Reagenzglas gebracht, worauf 2 ml Gummi-arabicum-Lösung hinzugefügt werden.

Standardprobe: 4 ml Standard I und 4 ml Standard II werden in je ein Reagenzglas geschüttet. Zu den Proben werden je 2 ml Fällungslösung und 4 ml destilliertes Wasser hinzugefügt. Sorgfältiges Mischen.

Sowohl die Blutprobe als auch die Standardprobe werden mit je 5 ml Nessler'schem Reagens versetzt, worauf man im Kolorimeter vergleicht. Kommt die Standard-I-Probe zur Verwendung, und wird sie auf 20 eingestellt, so findet die Berechnung in folgender Weise statt:

$$\frac{10 \times 1\,000 \times 20 \times 0.04}{8 \times 2 \times P} - B = \text{mg } \overset{+}{\text{UrN}} \text{ pro 100 ml Blut;}$$

$$\frac{500}{P} - B = \text{mg } \overset{+}{\text{UrN}} \text{ pro 100 ml Blut;}$$

P = An der Probe am Kolorimeter abgelesener Wert.

B = Blindwert in mg % N.

Vor allem auf Grund der Gegenwart von Gummi-arabicum-Lösung muss jedesmal, wenn diese Lösung frisch bereitet worden ist, die Blindwertbestimmung vorgenommen werden. Dieser Blindwert, der im allgemeinen nicht über 1—2 mg % beträgt, wird in folgender Weise bestimmt: In ein Reagenzglas, das 4 ml Standard I enthält, werden 2 ml Gummi-arabicum-Lösung, 2 ml Fällungslösung und 2 ml destilliertes Wasser geschüttet. Es wird eine Standard I enthaltende Standardprobe bereitet (siehe oben!). Beiden Reagenzgläsern werden je 5 ml Nessler'sches Reagens zugesetzt, worauf gemischt wird. Die Standard-I-Probe

wird auf 20 mm eingestellt, und dann die Blindprobe dagegen kolorimetriert, wobei der Blindwert in folgender Weise erhalten wird:

$$1\,000 \left(\frac{20 \times 0.04}{P_1} - 0.04 \right) = B \text{ in mg \% N;}$$

P_1 = an der Blindprobe am Kolorimeter abgelesener Wert.

Bei Werten des Harnstoffstickstoffs von über 80 mg % findet die Ablesung gegen die Standard-II-Probe statt, und die Berechnung wird dann folgende sein:

$$\frac{10 \times 1\,000 \times 20 \times 0.16}{8 \times 2 \times P} - B = \text{mg } \overset{+}{\text{UrN}} \text{ pro 100 ml Blut;}$$

$$\frac{2\,000}{P} - B = \text{mg } \overset{+}{\text{UrN}} \text{ pro 100 ml Blut;}$$

Bei der Berechnung werden die zugeführte Blutmenge (0.2ml) und die Ureasemenge (2 Tropfen) nicht berücksichtigt. Das Vernachlässigen dieser Faktoren führt zu einem Fehler von etwa 2 %, was sich jedoch im endgültigen Werte kaum bemerkbar macht.

Die Ureasemenge wurde möglichst klein gewählt (2 Tropfen einer 10 %igen Lösung), da es sich herausgestellt hat, dass selbst frisch bereitete Ureaselösungen nicht ganz unbedeutende Mengen NH_3 enthalten. Diese geringe Ureasemenge hat sich jedoch selbst für die grössten im Blute denkbaren Harnstoffmengen als genügend erwiesen, und ihr eigener NH_3 -Gehalt kann gänzlich vernachlässigt werden, falls die Lösung jedesmal, wenn eine Bestimmung vorgenommen werden soll, frisch bereitet wird. Selbst wenn die Lösung im Eisschrank aufgehoben wird, steigt ihr NH_3 -Gehalt ziemlich rasch.

Während man bei der Methode zur Reststickstoffbestimmung mit 0.1 ml Blut arbeiten kann, hat es sich hier als notwendig erwiesen, 0.2 ml Blut zu verwenden, um bei den normal vorkommenden etwa 15 mg % $\overset{+}{\text{UrN}}$ mit dem Nessler'schen Reagens eine für die Kolorimeterbestimmung geeignete Farbenstärke zu erhalten.

Als Schutzkolloid hat sich Gummi arabicum als überaus wirksam erwiesen. Seine Gegenwart bringt es jedoch mit sich, dass, wie oben erwähnt, eine Blindwertbestimmung vorgenommen werden muss.

Was die Normalwerte des Harnstoffstickstoffs im Blute anbelangt, sind diese, nach Angabe mehrerer Autoren, nicht nur individuell verschieden, sondern schwanken auch beim gleichen Menschen innerhalb gewisser Grenzen. So geben z. B. CAMERON und GILMOUR (3) 8—20 mg % an, TRUMPER und CANTAROW (4) 12—15 mg %. MACKAY und MACKAY (5) finden bei Frauen niedrigere Werte (5.14—18.2 mg %) als bei Männern (12.05—21.6 mg %). Ein Normalwert von 9—15 mg % dürfte jedoch der am häufigsten angenommene sein.

Normalerweise macht der Harnstoffstickstoff 50—60 % des Reststickstoffwertes aus. Bei Niereninsuffizienz wird jedoch verhältnismässig mehr Harnstoffstickstoff als andere Reststickstoffbestandteile zurückgehalten, wodurch seine Menge auf über 60 % steigt. Besonders bei leichteren Graden von Niereninsuffizienz dürften deshalb Parallelbestimmungen des Reststickstoffs und des Harnstoffstickstoffs von gewissem Wert sein. Wenn man, wie hier, bei beiden Bestimmungsmethoden im grossen Ganzen mit den gleichen Lösungen arbeitet, bringt dieses den Vorteil mit sich, dass die Werte untereinander besser vergleichbar sein werden.

Tabelle I.

Mit den hier angegebenen Methoden ausgeführte Parallelbestimmungen des Reststickstoffs und des Harnstoffstickstoffs.

	RN mg %	$\frac{1}{2}$ UrN mg %	$\frac{\frac{1}{2} \text{ UrN} \times 100}{\text{RN}}$
1	26	14	53.8
2	29	14.5	50.0
3	32	18	56.2
4	62	45.5	73.3
5	33	14	42.4
6	35	15	42.8
7	34	17	50.0
8	151	116	76.8
9	32	13	40.6

Um von der Zuverlässigkeit der Methode ein Bild zu bekommen, stellte man folgenden Kontrollversuch an: Je 0.1 ml Serum mit bekanntem Gehalt an Harnstoffstickstoff (14.1 mg %) wurde in Zentrifugengläser gebracht, die je 4 ml Molybdatlösung enthielten,

in der verschieden grosse, bekannte Mengen Harnstoff gelöst waren. Die Proben wurden in der Folge in völliger Übereinstimmung mit dem oben beschriebenen Verfahren behandelt, nur mit dem Unterschiede, dass hier nur die halbe Menge Molybdatlösung und Fällungslösung zur Verwendung kam.

Tabelle II.

Berechnete Menge hinzu- gegebenen + UrN, mg %	Gefundene Menge + UrN, mg %	Gefundene Menge + UrN, mg % abzüg- lich der im Serum vorhandenen 14.1 mg % + UrN	Abweichung in	
			mg %	%
22.4	35.9	21.8	0.6	2.7
22.4	36.3	22.2	0.2	0.9
22.4	36.1	22.0	0.4	1.8
22.4	36.3	22.2	0.2	0.9
57.4	71.1	57.0	0.4	0.8
57.4	70.4	56.3	1.1	2.3
57.4	70.1	56.0	1.4	3.0
57.4	71.4	57.3	0.1	0.2
80.8	94.0	79.9	0.9	1.5
80.8	94.9	80.8	0	0
80.8	94.3	80.2	0.6	0.85
80.8	94.0	79.9	0.9	1.3
150.8	168.7	154.6	3.8	2.7
150.8	166.3	152.2	1.4	1.0
150.8	165.1	151.0	0.2	0.14
150.8	165.1	151.0	0.2	0.14
290.8	300.6	286.5	4.3	1.6
290.8	300.6	286.5	4.3	1.6
290.8	304.8	290.7	0.1	0.03
290.8	307.0	292.9	2.1	0.75

Zusammenfassung.

Es wird eine einfache Methode zur Bestimmung des Harnstoffstickstoffs an 0.2 ml Blut beschrieben. Ihr Vorzug besteht darin, dass das Blut in eine Lösung gebracht wird, die gleichzeitig als eine für optimale Ureasewirkung geeignete Pufferlösung und nach leichter Ansäuerung als Eiweissfällungsmittel dient. Man erhält

ein eiweissfreies Zentrifugat und nimmt darauf, in Gegenwart von Gummi arabicum als Schutzkolloid, nach direkter Nesslerisierung eine kolorimetrische Bestimmung vor.

Für einen Geldbeitrag zur Ausführung der Arbeit möchte ich der »Kungl. Fysiografiska Sällskapet« in Lund meinen Dank aussprechen.

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In vitro Studies on the Role of Vitamin D in the Metabolism of Calcium and Phosphorus in the Rat Bones.¹

By

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The object of the present work, which forms a part of a more extended research on the mineral metabolism of the animal organism, was to elucidate, on the basis of *in vitro* experiments, the influence of vitamin D on the calcium and phosphorus metabolism in the animal organism.

Although the literature concerning the relation of vitamin D to the metabolism of calcium and phosphorus is very extensive, up to the present only a few studies have been recorded which try to explain, with *in vitro* experiment, the role of vitamin D in the processes of ossification, a question which still lacks more detailed information.

Among the communications found in literature must be mentioned the observations made with *in vitro* experiments by SHIPLEY, KRAMER and HOWLAND (1925, 1926) that ossification may occur in the rachitic cartilage, if the surrounding incubation solution contains a suitable amount of calcium and phosphorus. ROBISON and SOAMES (1930) mention that rachitic cartilage calcifies in a regular manner, if placed in solutions of inorganic salts supersaturated with respect to the bone salt, as well as in lower concentrations of calcium and phosphorus if organic phosphoric ester is present. Accordingly, this would indicate that rachitis is a disease of blood and not of the bones. In later studies *in vitro* ROBISON and ROSENHEIM (1934) established that addition of

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vitamin D into the incubation solution had no histologically demonstrable action on the calcification of the normal bone. On the other hand, however, ROSENHEIM (1934), mentions that in the *in vitro* experiments the calcifying power of the cartilage of rats decreases with increasing periods on a rachitogenic diet. There is also a short communication by FLEISCHMANN (1937) that, according to a histological study, the calcification of the bird bone occurs at a slower rate in the serum plasma of rachitic animal than in the normal plasma and that an addition of calcium and phosphorus salts into the rachitic plasma does not suffice to compensate fully the deficiency of vitamin D.

The experiments *in vitro* that have been performed with a technique of more chemical nature, have chiefly dealt with the question of the resorption of calcium and phosphorus from the intestine. Some time ago NICOLAYSEN (1937) concluded from his *in vitro* experiments that vitamin D promotes the resorption of calcium from the intestine, but not the resorption of phosphorus. In other tissues no difference seemed to exist in the absorption of calcium by normal and rachitic animals. HARRIS (1932) had already earlier advanced a theory that rachitis involves a failure in the "net absorption" of calcium and phosphorus.

The above experiments for the elucidation of the rôle of vitamin D were made only with organs of animals suffering from a deficiency of vitamin D. Literature does not report chemical investigations which would give more information based on experiments *in vitro* concerning the metabolism of calcium and phosphorus in D-hypervitaminotic animals.

With regard to the lack of experiments *in vitro* concerning D-hypervitaminosis we considered it reasonable to take this very point for the chief object of our investigations which were started in 1936. Therefore we used in the first experiments animals in which D-hypervitaminosis had been induced by large amounts of vitamin D. Somewhat later we began to arrange, together with experiments on D-hypervitaminosis, also experiments with D-avitaminotic animals. The main purpose of all these experiments was to find out whether it is possible to demonstrate, with experiments *in vitro* under suitable conditions, the effect of vitamin D upon the ability of bone, firstly, to take up, and secondly, to retain calcium or phosphorus.

In the following are presented the results of some of the preliminary experiments made exclusively with rat bones.

Methods.

The experimental material consisted generally of preparations obtained from the fore and hind legs of rats. In the experiments on D-hypervitaminosis adult animals were used, the weight of which varied from 150 to 210 g. The fore leg preparations (humerus, radius and ulna together) weighed from 450 to 700 mg, the hind leg preparations (femur and tibia) from 800 to 1 600 mg. The rats in the experiments on D avitaminosis weighed from 40 to 80 g, their fore leg preparations from 300 to 800 mg and the hind leg preparations from 700 to 1 500 mg.

D-hypervitaminosis was produced by administration of varying amounts of "vigantol" to the rats by means of a stomach tube, the daily dosage varying from 12 000 to 36 000 I. U. The control animals received the corresponding amount of vitamin D-free sesame oil. The basal diet was otherwise the same in both groups.

In the experiments on D-hypovitaminosis the diets of MAC COLLUM No. 3143 and STEENBOCK No. 2965 were used for the production of rachitis. The control animals were kept free from rachitis by giving them daily small doses of strongly diluted "vigantol".

The bone preparations were made as follows. Immediately after killing of the animal the leg was carefully removed from the surrounding tissues, care being taken to prevent damage of cartilage and periosteum. The bone preparation was then placed in a 20 cc. test tube, which contained 10 cc. calcium chloride, sodium phosphate or physiological sodium chloride solution respectively. In experiments on the uptake of calcium we used in the incubation calcium chloride solution containing 10—12 mg % calcium and respectively, for the phosphor investigations sodium phosphate solution containing 10—100 mg % phosphorus. In both cases a suitable addition of sodium chloride was made into the incubation solution to bring about isotonicity. In examining the excretion of calcium and phosphorus from the bone 0.85 % sodium chloride solution was employed as the incubation medium.

The bone preparation was incubated at room temperature for 24 hours.

In the analysis of the incubation solution the well-known titrimetric micromethod of KRAMER-TISDALL proved the most

suitable. In the calcium oxalate precipitation it was found necessary, however, to prolong the time of precipitation to 24 hours in order that uniform values could be obtained. The determinations of phosphorus were carried out photometrically by FISKE-SUBBAROWS method. Traces of albumin were removed prior to phosphor determinations with 7 % trichloroacetic acid.

Results.

I. Uptake and retention of calcium and phosphorus in normal rat bones.

Before entering upon the actual vitamin experiments it seemed advisable to investigate in general the behaviour of normal bone in the uptake and retention of calcium and phosphorus, a problem which, in view of the renewal of bone, is not devoid of interest but which has not been more closely investigated with chemical methods.

1. *Uptake of calcium.*

The following table summarises the results of six experiments. In three of them was studied the calcium absorption of fore leg preparations of varying weight and in the other three that of the hind leg preparations.

Table 1.

Bone preparation	Weight of bone preparation, mg	Calcium uptake, mg
Fore leg	500	0.12
'	600	0.06
'	890	0.05
Hind leg	950	0.17
'	1,150	0.10
'	1,150	0.06

It can be seen from the table that the normal rat bone has the ability to take up calcium from the incubation solution. The analytical differences are slight but, nevertheless, convincingly outside the limits of error of calcium analyses.

2. Retention of calcium.

The following table gives the results of experiments performed with the same amount of bone preparations from bones of approximately the same weight as above, with the exception that calcium-free physiological sodium chloride solution was used as incubation solution.

Table 2.

Bone preparation	Weight of bone preparation, mg	Loss of Calcium, mg
Fore leg	550	0.40
'	570	0.52
'	650	0.55
Hind leg	1,100	0.65
'	1,300	0.58
'	1,450	0.61

The table shows that a distinct excretion of calcium occurs from the normal bones into the surrounding solution. Regarding the weight of the preparations it is evident that the fore leg preparations have given pronouncedly largest amounts calcium per weight unit into the solution.

3. Uptake of phosphorus.

In the experiments concerning the uptake of phosphorus an incubation solution containing 10 mg % phosphorus was first used. No uptake of phosphorus from the solution could be observed here as shown by the values of the four experiments recorded in table 3; on the contrary the phosphorus content of the incubation solution was higher after the incubation than before it. In later experiments (in collaboration with ANTELL and BARDY) higher concentrations of phosphorus up to 100 mg % were used. Nevertheless, even in the highest concentrations no decrease was noted in the phosphorus content of the incubation solution. On the contrary, phosphorus seemed to be excreted, as in the above mentioned phosphorus experiments, from the bone into the incubation solution.

Table 3.

Bone preparation	Weight of bone preparation, mg	Phosphorus uptake, mg
Fore leg	475	— 0.19
'	550	— 0.85
Hind leg	1,100	— 0.12
'	1,150	— 0.31

4. Retention of phosphorus.

In Table 4 are compiled the results of experiments performed with preparations from fore and hind legs under equal conditions as those above concerning the calcium retention using physiological sodium chloride as the incubation solution.

Table 4.

Bone preparation	Weight of bone preparation, mg	Loss of Phosphorus, mg
Fore leg	550	0.26
'	570	0.38
'	650	0.33
Hind leg	1,100	0.52
'	1,300	0.38
'	1,400	0.36

It can be seen that a pronounced transfer of phosphorus from the bone preparations into the incubation solution occurs in all cases. It is difficult to conclude from these few preliminary experiments which factors cause and influence this transfer of phosphorus. Apart from the purely physical factors there is a possibility that the transfer of phosphorus into the solution is in some relation to the activity of the bone phosphatases. We are going to examine later in detail the excretion of phosphorus into the solution.

In any case, the preliminary studies *in vitro* with normal bone preparations showed that between the bones and the solution an exchange of calcium and phosphorus occurs which is distinctly

demonstrable by chemical methods. The technique employed makes possible the investigation of the calcium and phosphorus metabolism in the bones under different conditions.

II. Uptake and retention of calcium and phosphorus in the D-hypervitaminosis.

1. Uptake of calcium.

Table 5 summarises the results of parallel experiments, concerning the calcium uptake, carried out with fore leg preparations of D-hypervitaminotic and control animals. The hypervitaminotic animals had received, during three days, 24,000 I. U. daily, the control animals receiving sesame oil only.

Table 5.

Bone preparation	Weight of bone preparation, mg	Calcium uptake, mg
	Controls	
Fore leg	600	0.08
'	625	0.08
'	720	0.06
'	720	0.02
	Hypervitaminotic animals	
'	500	0.31
'	640	0.15
'	672	0.19
'	680	0.19

A comparison of the calcifying power of bone preparations of approximately the same weight from D-hypervitaminotic and control animals shows that liberal administration of vitamin D has distinctly increased the ability of the bones to absorb calcium from the solution. The mean value for calcium absorption in the normal cases is only about one-fourth of the corresponding value in the hypervitaminotic cases. The experiments support the view that vitamin D somewhat makes the bones "more hungry" for calcium than usual.

2. Retention of calcium.

The influence of vitamin D on the uptake of calcium led to the assumption that this vitamin also increases the ability of the bones to retain calcium. In order to elucidate this point a series of experiments were made, the results of which are recorded in Table 6. The production of hypervitaminosis in these cases was accomplished by administering daily 12,000 I. U. of vitamin D during four days. The experimental material in the incubation experiments consisted of hind leg preparations.

Table 6.

Bone preparation	Weight of bone preparation, mg	Loss of Calcium, mg
	Controls	
Hind leg	1,090	0.56
"	1,100	0.65
"	1,195	0.44
"	1,300	0.58
	Hypervitaminotic animals	
"	1,205	0.44
"	1,320	0.60
"	1,370	0.58
"	1,650	0.50

A comparison of the results in both groups shows no such difference in the loss of calcium as in the above experiments on the uptake of calcium. It is difficult to draw conclusions from the results, as the weight of the bone preparations used in the control experiments was, on the average, lower than in the experiments on D-hypervitaminosis. The variations in both groups are, however, so great that the final solution of the problem seems possible only by means of an extensive test material. (The supplementing experiments carried out so far, showed in some cases a distinct tendency for a decrease of calcium excretion in D hypervitaminosis.)

3. Retention of phosphorus.

Table 7 gives data from experiments concerning the loss of phosphorus when hind leg preparations of D-hypervitaminotic animals and the controls were kept in phosphorusfree sodium chloride solution. In these cases vitamin D was administered in daily doses of 12,000 I. U. during four days.

Table 7.

Bone preparation	Weight of bone preparation, mg	Loss of Phosphorus, mg
Controls		
Hind leg	1,090	0.84
'	1,100	0.52
'	1,195	0.38
'	1,300	0.88
Hypervitaminotic animals		
'	1,205	0.31
'	1,325	0.29
'	1,370	0.29
'	1,650	0.24

It can be seen from the table that a difference exists between the values for phosphorus excretion in D-hypervitaminosis and controls, the excretion being lower in the former group. Accordingly vitamin D would somehow be able to effect a retention of inorganic phosphorus in the bone. It may be remarked against the arrangement of the experiments that, due to the different weight of the bones, the groups are not fully comparable to each another. Considering, however, also the above-reported values for the retention of phosphorus in the control animals (Table 4), the values for excretion lead to the conclusion that the effect is ascribable to vitamin D.

III. Uptake and retention of calcium and phosphorus in rachitis.

Following the above observations on D-hypervitaminosis, our chief attention was directed to the possible effects caused by D-

hypovitaminosis under the same experimental conditions. The rats employed in the experiments showed typical symptoms of rachitis as a result of prolonged chronic deficiency of vitamin D. By chemical examination a pronounced decrease of inorganic phosphorus was discernable in the serum.

1. Uptake of calcium.

Table 8 gives the values of calcium uptake obtained with the hind leg preparations of two rachitic and two normal rats. It is noteworthy that neither of the hind leg preparations of the one rachitic rat has taken up calcium from the solution, on the contrary, they have given off calcium into the solution. Thus the phenomenon concerned would be completely contrary to that in D-hypervitaminosis in which calcium uptake was increased. The changes in the calcium content of the solution caused by the preparations of the other rachitic rat are, however, in accordance with those in the control animals. For the final settlement of this point more experiments with a more extensive material are naturally needed.

Table 8.

Bone preparation	Weight of bone preparation, mg	Calcium uptake, mg
Controls		
Fore leg	470	0.14
„	460	0.18
„	700	0.02
„	750	0.02
Rachitic animals		
„	620	— 0.13
„	650	— 0.22
„	450	0.11
„	450	0.13

2. Retention of calcium.

A comparison of the values of calcium uptake, obtained with the bones of rachitic rats (Table 9) and the corresponding values of normal animals, with those of the previous experiments on D-

hypervitaminosis show that there is an apparent tendency towards reduced ability to retain calcium in the rachitic cases. This result is again contrary to what was the case in D-hypervitaminosis.

Table 9.

Bone preparation	Weight of bone preparation, mg	Loss of Calcium, mg
Controls		
Fore leg	340	0.48
"	300	0.45
"	500	0.47
"	470	0.93 (?)
Rachitic animals		
"	380	0.62
"	360	0.60
"	300	0.96
"	320	0.97

3. Retention of phosphorus.

It is evident from Table 10, which gives the values obtained with bone preparations of rachitic and control animals, that no distinct difference can be observed between the two groups.

Table 10.

Bone preparation	Weight of bone preparation, mg	Loss of Phosphorus, mg
Controls		
Fore leg	380	0.38
"	420	0.27
"	500	0.24
"	470	0.24
Rachitic animals		
"	380	0.27
"	360	0.30
"	300	0.27
"	320	0.20

Direct comparison of the results is rendered difficult by the fact that the bones of the control animals have, on the average, been bigger than those of the rachitic animals. If the amount of excreted phosphorus is calculated per weight unit of bones, it appears that in rachitis the amount of excreted phosphorus per weight unit of bone is somewhat greater than in the controls.

Discussion of the results.

The preliminary experiments which were carried out in order to obtain a basis for subsequent vitamin experiments, brought into light facts that are not devoid of interest in view of the mineral metabolism of the bone. It appeared from the experiments that the bone is able to take up calcium from the surrounding solution containing calcium. On the other hand, if the bone is incubated in the calciumfree solution it gives up calcium into the solution. With regard to inorganic phosphorus, the bone did not take it up although the phosphate concentration was raised high. On the other hand, when the bone was incubated in solutions with or without phosphorus, inorganic phosphorus always appeared.

An explanation to these variations is naturally offered by the simple physical diffusion. On the other hand, the possibility is not excluded that some active function of cells plays a part in these phenomena. These points are further investigated in new experiments now in progress. It must be mentioned in this connection that the experiments of ROBISON on the ossification had proved that the bone does not take up phosphorus in inorganic form, but as phosphoric acid esters.

Our experiments showed that D-hypervitaminosis is characterised by an increased ability of the bones to uptake calcium when the incubation takes place in solutions containing calcium, and by a decreased tendency to give up calcium into the surrounding Ca-free solution. At the same time, the excretion of inorganic phosphorus into the phosphorus-free incubation solution showed a tendency to decrease. On the other hand, in a deficiency of vitamin D a decrease was noted in the uptake of calcium, and an increased tendency towards an excretion of calcium into the calciumfree incubation solution. Through the effect of vitamin D the bone would accordingly become somehow more hungry for calcium and also attain an increased ability to retain calcium and possibly also phosphorus.

It was mentioned already that the way of action of vitamin D on the bone — whether direct or indirect — has not been definitely settled up till present. It has been assumed that the changes in the bone, specific to rachitis, are merely consequences of the disturbances in the calcium and phosphorus of the serum somehow caused by vitamin D. The observations described above indicate that vitamin D has a direct influence on the bone. This does not mean, that the effect of vitamin D could not appear in other tissues. It is conceivable that the ability of the cells in general — or at any rate of certain groups of cells — to take up calcium or retain it, is somehow dependent on vitamin D. Some experiments with other tissues, carried out in connection with this work have to some extent revealed that also in other tissues it is possible to bring about phenomena similar to those described above. That the resorption of calcium from the intestine is disturbed by vitamin D deficiency has been earlier established, as mentioned above.

Summary.

The object of the work has been to investigate, by means of experiments *in vitro*, the effect of vitamin D on calcium and phosphorus metabolism in rat.

The normal bone has the ability to take up calcium from the incubation solution under certain conditions and, on the other hand, to give up calcium into the calcium-free incubation solution.

The bone was not able to take up inorganic phosphorus in the incubation experiments. On the other hand the excretion of inorganic phosphorus into the solution could always be demonstrated — both in incubation solutions with and without phosphorus.

The bones of rats which had received large amounts of vitamin D seemed to be able to take up relatively more calcium than the bones of normal rats. On the other hand it is possible that the bone is then to some extent more than normally able to take up calcium and phosphorus.

With regard to the deficiency of vitamin D, a tendency towards a decreasing uptake of calcium and increased loss of calcium was noted.

The experiments support the view that vitamin D has a direct influence upon the mineral metabolism of the bone.

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Über den Einfluss lokaler Hautschädigungen (mechanische Verletzung, Erfrierung, Verbren- nung) auf die periphere Blutverteilung.¹

Von

GÖSTA von REIS und FRITIOF SJÖSTRAND.

(Mit 2 Figuren im Text.)

In früheren Arbeiten (G. von REIS und F. SJÖSTRAND 1937, 1938, 1940) sind die Resultate von Untersuchungen über den Einfluss verschiedener chemischer und physikalischer Hautreize auf die Blutmenge in peripheren Blutgefäßen der Leber und Nierenrinde veröffentlicht worden. Bei mehreren Reizmitteln war dabei eine erhebliche Zunahme der Blutmenge in diesen Gefäßen konstatiert worden.

Bei einer ersten Analyse des Zustandekommens dieses Effekts hat sich derselbe als von einer intakten Hautinnervation abhängig erwiesen. Irgendein Einfluss durch Resorption von Substanzen aus der Haut, welche auf die peripheren Blutgefäße einwirken könnten, hat sich dagegen nicht feststellen lassen.

Die Absicht bei den in der vorliegenden Arbeit beschriebenen Versuchen war in erster Linie zu untersuchen, inwieweit eine lokale Hautschädigung durch z. B. Verbrennung, Erfrierung oder mechanische Einwirkung einen Einfluss auf die peripheren Blutgefäße in inneren Organen ausübt, von welchen bisher Leber und Nierenrinde, bei Verbrennung auch der M. masseter, untersucht wurden. Hierdurch würde die Anschauung ziemlich direkt gestützt werden, dass diese Blutgefäße primär einen beitragenden Faktor bei der Entstehung des Schocks nach ausgedehnten derartigen Schädigungen darstellen.

¹ Der Redaktion am 14. September 1940 zugegangen.

Ausserdem suchten wir mehr allgemein zu entscheiden, ob bei einer Wärmeschädigung in der Haut Substanzen gebildet oder freigemacht werden können, welche nach ihrer Resorption imstande sind, auf die peripheren Blutgefässe im Sinne der Annahme von LEWIS (1927) einzuwirken. Zu diesem Zweck haben wir bei lokalen Hautschädigungen den Effekt, welcher an den peripheren Blutgefässen resultiert, wenn die Schädigung Haut mit intakter Innervation trifft, mit demjenigen einer entsprechenden Schädigung von denervierter Haut verglichen.

Während über die verschiedenen Wirkungen der Verbrennung ein umfangreiches Schrifttum entstanden ist sind Angaben über den Effekt von Erfrierungen nur spärlich vertreten, und, soweit wir finden konnten, experimentelle Arbeiten über die diesbezüglichen Folgen mechanischer Hautschädigungen überhaupt nicht vorhanden. Hinsichtlich der Verbrennung wird auf Monographien und Übersichtsreferate von MARCHAND (1908), STOCKIS (1913), WILSON (1929) und HARKINS (1938) verwiesen.

Es ist vor allem die Ursache des in engem Zusammenhang mit ausgedehnten Brandwunden auftretenden Schocks, welche man zu analysieren suchte, und es handelte sich dabei im grossen ganzen darum, zu entscheiden, ob dieser durch eine Einwirkung auf das Nervensystem zustandekommt — welche entweder eine Hemmung innerhalb verschiedener vegetativer Zentra in der Medulla oblongata oder eine übermässige Reizung der Nebennieren herbeiführen könnte, durch die der Adrenalinvorrat dieses Organs erschöpft würde, mit darauf folgender allgemeiner Gefässdilatation —, durch eine Vergiftung mit Substanzen aus dem geschädigten Gewebsgebiet, durch Plasmaverlust infolge von Ödemen, oder schliesslich durch Bakterienwirkung.

Mehrere Autoren (DOHRN 1901, WILMS 1901, STOCKIS 1903) haben die Ansicht geäussert, dass der Schock wahrscheinlich einem Zusammenwirken mehrerer Faktoren zuzuschreiben sei. Heutzutage scheint man jedoch allgemein eher einen Effekt durch Intoxikation als den dominierenden Faktor anzunehmen, wenn sich auch eine sichere Stütze für diese Erklärungsweise nicht anführen lässt.

Seitdem LEWIS (1927) und KROGH (1929) auf die Möglichkeit hingewiesen haben, dass der Schock durch Histamin- oder H-Substanzvergiftung zustandekommen sollte, glaubten mehrere Forscher, experimentell gewisse Anhaltspunkte für diese Hypothese finden zu können, während andere beträchtliche Unter-

schiede zwischen Histamin- und Verbrennungsschock feststellten.

Die Versuche, den Verbrennungsschock mit dem Histaminschock gleichzusetzen, sind ein Ausdruck der Auffassung des ersteren als Folge einer Kreislaufsinsuffizienz, u. a. auf Grund einer hochgradigen Dilatation der peripheren Blutgefäße.

Auf das Vorliegen einer solchen Dilatation hat man u. a. aus Blutdruckversuchen geschlossen (SONNENBURG 1878, STOCKIS 1903 u. a. m.). SIMONART (1930) beobachtete ausserdem die Farbe der Organe, nachdem die Versuchstiere durch Verblutung an verschiedenen Zeitpunkten nach einer in Äthernarkose hervorgerufenen ausgedehnten Verbrennung ($\frac{2}{3}$ der Körperoberfläche) getötet worden waren. Er fand dabei, dass vor allem die Bauchorgane — besonders die Nieren — bei den Tieren mit Brandwunden röter waren als bei den Kontrolltieren. Bei in Einzelfällen vorgenommener mikroskopischer Untersuchung sah S., dass die Kapillaren stark erweitert waren.

Die Allgemeinwirkung von Erfrierung und Auftauung eines Gewebsgebiets ist nur in einer geringen Anzahl experimenteller Arbeiten behandelt worden. PFEIFFER (1927) gibt an, dass der Effekt dieser Art von Gewebsschädigung dem bei Verbrennung entspreche und auf Resorption von Zerfallsprodukten aus dem geschädigten Gewebsgebiet beruhe. Experimentell wird diese Behauptung nicht gestützt.

Dagegen hat HARKINS gemeinsam mit verschiedenen Mitarbeitern (1934, 1935, 1937) nachgewiesen, dass man bei Hunden durch Kälteschädigung einen Schockzustand hervorrufen kann, welcher dem bei Verbrennung in mehreren Beziehungen ähnlich ist (Bluteindickung durch Plasmaverlust, herabgesetztes Blutungsvolumen). Wie dieser Schockzustand ausgelöst wird scheint nicht Gegenstand der Untersuchung gewesen zu sein, obwohl man hierin ein gewisses prinzipielles Interesse erblicken könnte.

Methodik.

Da die allgemeine Versuchsanordnung bei diesen Versuchen gänzlich mit derjenigen übereinstimmt, über welche wir in unseren früheren Arbeiten berichtet haben, verweisen wir auf diese (1938, 1940).

Als Versuchstiere haben wir Meerschweinchen mit einem Gewicht von 400—600 g verwendet. Tiere beider Geschlechter fanden in ungefähr gleichem Masse Verwendung, und eine an das Geschlecht gebundene Variation wurde nicht beobachtet.

Das behandelte Hautgebiet war 3×6 bis 4×6 cm gross und erstreckte sich quer über die Bauchhaut; die kaudale Grenze lag in der Höhe der Nabelebene.

Die Versuche wurden in Pernoctonnarkose vorgenommen.

Die Tiere wurden durch Zerquetschung des Halsmarks getötet.

Die Blutmenge in den peripheren Blutgefässen wurde nach T. SJÖSTRAND (1934) und VON REIS, SILFVERSKIÖLD, F. SJÖSTRAND und T. SJÖSTRAND (1938) bestimmt, d. h. die Anzahl roter Blutkörperchen pro mm³ Gewebe in der Leber und Nierenrinde wurde nach selektiver Färbung von Mikrotomschnitten durch Vergleich unter dem Vergleichsmikroskop mit einer Serie von Standardpräparaten festgestellt. Bei Verbrennungsversuchen wurde ausserdem die Anzahl offener Kapillaren pro mm² des Querschnitts durch den M. masseter durch Zählung bestimmt.

Eine oberflächliche Verbrennung wurde auf zwei verschiedene Arten hervorgerufen, teils durch Brennen mit einem 0.1 mm dicken Messingblech oder einem 0.05 mm dicken Kupferblech, welches zum Glühen gebracht und dann zwei- bis dreimal 2—4 Sek. gegen die Haut gepresst wurde, teils durch intensive Bestrahlung mit einer Wärmelampe. Durch die geringe Wärmekapazität der dünnen Metallbleche wurde die Verbrennung sehr oberflächlich. Die von uns verwendete Wärmelampe (»Glory«) sendet Wärmestrahlen von sowohl höherer wie niedrigerer Wellenlänge aus. Die Tiere wurden mit derselben 5—10 Min. intensiv bestrahlt, wobei eine deutliche Verbrennung zustandekam.

In denjenigen Fällen, wo die Verbrennung mittels Metallblechs hervorgerufen worden war, wurden die Tiere 45 Min. nach dem Eingriff getötet, während sie bei den Versuchen mit der Wärmelampe 35—40 Min. nachdem die Hautveränderungen sich entwickeln konnten getötet wurden.

Eine lokale Kälteschädigung wurde entweder durch Bespritzen mit Chloräthyl erzeugt oder dadurch, dass ein mit Kohlensäureschnee gefüllter Kupferbehälter von geeigneter Form gegen die Haut gepresst wurde.

Die Haut wurde in beiden Fällen 10—20 Min. in gefrorenem Zustand gehalten, wonach man sie auftauen liess. Ausserdem fand partielle Auftauung während der Bespritzung mit Chloräthyl und der Gefrierung mit Kohlensäureschnee statt, da es auf andere Weise unmöglich war, das Gefrieren auf die Haut zu beschränken.

Die Tiere wurden 30—45 Min. nach dem Auftauen getötet.

Eine mechanische Hautverletzung wurde entweder dadurch erzielt, dass die Haut mit einer Rasierklinge zerschnitten wurde oder durch Zerkratzen mit einer feinen Säge. Die Tiere wurden 45 Min. nach dem Eingriff getötet.

In denjenigen Fällen, wo wir einen deutlichen Effekt erhielten, wurde ein entsprechender Versuch an Tieren ausgeführt, deren Bauchhaut ihrer segmentalen Innervation beraubt worden war, nach derselben Methode, wie sie in früheren Arbeiten (1938) angegeben ist.

Kurze Beschreibung der erzielten Hautveränderungen.

Beim Brennen mit dem Messingblech wurde die Haut stark zusammengezogen und verdickt. Sie wurde hart und unelastisch, blasste ab und nahm teilweise eine bräunliche Färbung an. Von der Innenseite gesehen hat ein abpräpariertes Hautstück eine starke Gefässinjektion aufgewiesen. Ein Ödem im Corium wurde nicht beobachtet.

Die Haut war teilweise mit der darunterliegenden Muskulatur adhärent. Die Epidermis war etwas ödematös-verdickt, und die äussere Muskelschicht war entsprechend der Stelle der Verbrennung in geringerer Ausdehnung etwas gerötet und ödematös. Peritoneum und Därme waren unversehrt.

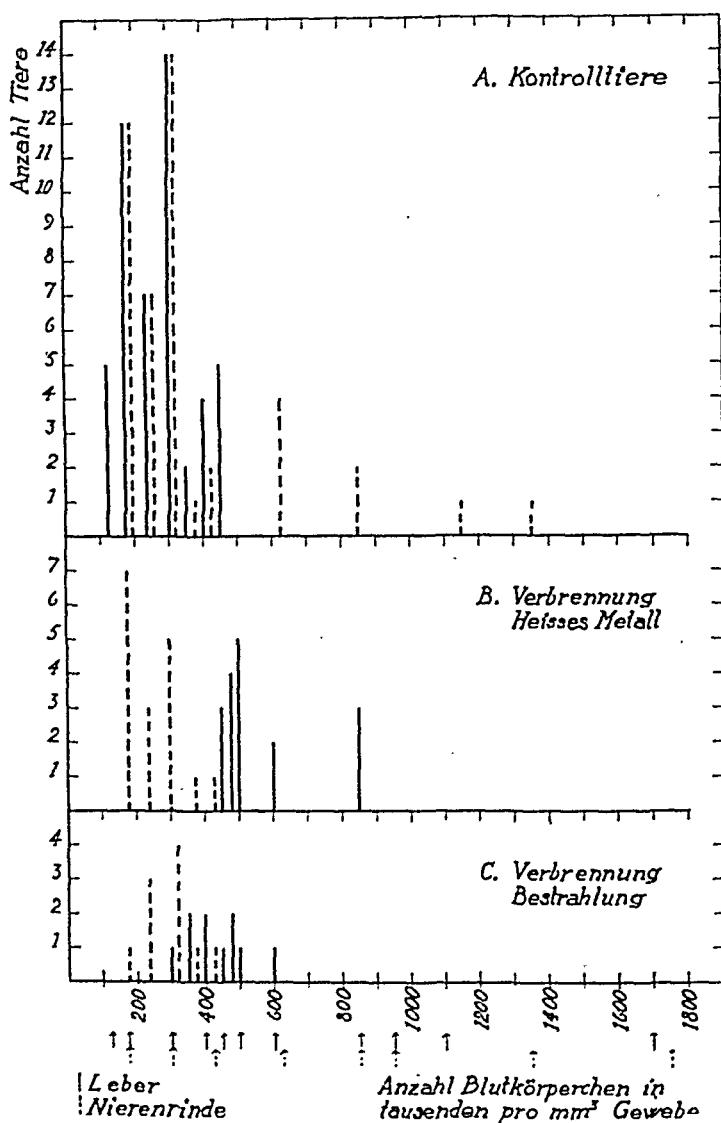
Die mit dem dünnen Kupferblech hervorgerufene Brandwunde war von geringerer Ausdehnung. Die Haut war auch hier, wenn auch in geringerem Grade, zusammengezogen und ihrer Farbe und Konsistenz nach verändert, ohne dass es jedoch, abgesehen von vereinzelt Fällen, zu einer Bräunung gekommen wäre. Die Haut liess sich leicht von der Unterlage ablösen, und eine Einwirkung auf die darunterliegende Muskulatur wurde nicht beobachtet. In einzelnen Fällen sah man ein unerhebliches Ödem im Unterhautzellgewebe.

Beim Brennen mit der Wärmelampe wurde die Haut zunächst gerötet, worauf sich innerhalb eines Bezirks mit nahezu kreisrunder Begrenzung und einem Durchmesser von 4—5 cm ein Ödem in der Haut bildete. Im Zentrum dieses ödematösen Gebiets blasste später die Haut ab, und das Ödem bildete einen Wall rings um die so entstandene abgeblasste Zone, welche einen Durchmesser von etwa 2 cm hatte. Im übrigen ähnelten die Veränderungen denjenigen beim Brennen mit Metallblech, wenn sie auch mehr in die Tiefe gingen, sodass die Muskulatur in grösserem Ausmass geschädigt wurde.

Bei keinem Fall wurde Blasenbildung in der Haut beobachtet.

Die Ödeme waren von unerheblicher Ausdehnung, sodass ein Einfluss auf den Flüssigkeitsgehalt des Blutes oder der Gewebe keine Fehlerquelle von Bedeutung bilden konnte.

Bei der Gefrierung der Haut mit Chloräthyl entstanden Veränderungen, welche in gewissem Masse denjenigen beim Brennen mit der Wärmelampe ähnlich waren. Zentral bildete sich also ein blasser Hautbezirk mit einer schwach-bräunlichen Verfärbung,



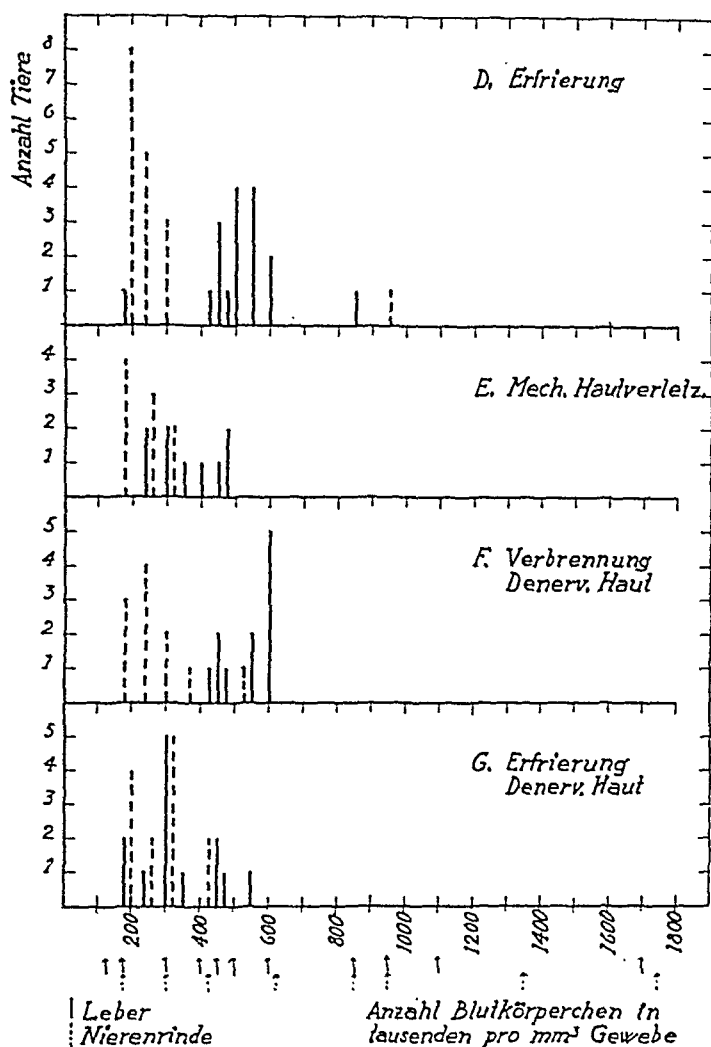
Die Pfeile unter der Abszisse bezeichnen die Werte bei den verwendeten Standardpräparaten.

Diagramm 1.

umgeben von einem weniger stark ausgeprägten Ödemwall. Die Haut war mit Ausnahme der anämischen Zone in dem verletzten Gebiet gerötet.

Nach Gefrierung mit gekühltem Metall wurde nur eine Rötung der Haut beobachtet.

Bei der mechanischen Hautverletzung mittels Rasierklinge



Die Pfeile unter der Abszisse bezeichnen die Werte bei den verwendeten Standardpräparaten:

Diagramm 2.

wurden nur oberflächliche kutane, dichtliegende Schnitte erzeugt. Die mit der Säge hervorgerufenen Verletzungen waren ebenfalls oberflächlich und beschränkten sich auf Epidermis und Corium. In beiden Fällen kam es nur zu unbedeutender Blutung.

Hinsichtlich der statistischen und graphischen Bearbeitung der erhaltenen Werte verweisen wir auf unsere frühere Arbeit über den Einfluss lokaler physikalischer und chemischer Hautreize auf die periphere Blutverteilung (1940).

Versuchsergebnisse.

Kontrolltiere.

Da wir mit denselben Versuchsbedingungen gearbeitet haben, wie sie von uns in früheren Arbeiten beschrieben sind, gelten die in diesen veröffentlichten Kontrolltierwerte für die periphere Blutmenge in der Leber und Nierenrinde sowie für die Anzahl offener Kapillaren im Querschnitt des *M. masseter* auch in diesem Zusammenhang.

Die Mittel der Werte für die periphere Blutmenge betrugen bei der Leber (49 Tiere) $M = 268,000$ und bei der Nierenrinde (44 Tiere) $M = 363,000$ Blutkörperchen pro mm^3 Gewebe. Diagramm 1 A lässt die Streuung der Primärwerte erkennen. Die Anzahl offener Kapillaren im Querschnitt des *M. masseter* war im Mittel (19 Tiere) $1,330$ pro mm^2 .

Die Primärwerte für die Anzahl offener Kapillaren im *M. masseter* von Kontrolltieren findet man in unserer früheren Arbeit über den Einfluss lokaler chemischer und physikalischer Hautreize auf die periphere Blutverteilung (1940).

Lokale Verbrennung.

Bei Versuchen mit Brennen mittels Messingblechs wurden die Mittelwerte (17 Tiere) für die Leber $M = 559,000$ und für die Nierenrinde $M = 253,000$ Blutkörperchen pro mm^3 Gewebe. Aus Diagramm 1 B wird die Streuung der Primärwerte ersichtlich. Im *M. masseter* erhielten wir im Mittel (15 Tiere) $1,650$ blutgefüllte Kapillaren pro mm^2 Querschnitt. Die Primärwerte findet man in Tab. 1.

Aus diesen Werten geht hervor, dass die periphere Blutmenge in der Leber auf das doppelte der entsprechenden Kontrolltierwerte gestiegen ist. In der Nierenrinde dagegen ging diese Blutmenge um 30 % zurück, eine Verringerung, welche jedoch für dieses Organ innerhalb der Fehlergrenzen der Methode liegen dürfte.

Die Anzahl offener Kapillaren pro Flächeneinheit im Querschnitt des *M. masseter* nahm um 25 % zu. Die Differenz der mittleren Fehler (t) zwischen dieser Serie und der Kontrolltierserie beträgt 8.4, weshalb die Differenz der beiden Mittelwerte statistisch gesichert ist.

Tabelle 1.

Anzahl offener Kapillaren pro mm² Querschnitt des M. masseter 45 Min. nach Erzeugung einer oberflächlichen lokalen Verbrennung.

Nr.	intakte Haut innervation	denervierte Haut
1	1,690 ± 53	1,330 ± 46
2	1,450 ± 47	1,340 ± 47
3	1,630 ± 52	1,430 ± 44
4	1,630 ± 34	1,180 ± 34
5	1,700 ± 37	1,520 ± 49
6	1,670 ± 44	1,250 ± 29
7	1,630 ± 43	1,430 ± 36
8	1,680 ± 39	1,300 ± 40
9	1,710 ± 47	1,310 ± 28
10	1,600 ± 45	1,520 ± 53
11	1,770 ± 45	1,450 ± 44
12	1,610 ± 37	
13	1,700 ± 51	
14	1,760 ± 48	
15	1,570 ± 39	
M =	1,650	1,370

Bei durch Bestrahlung mit der Wärmelampe erzielter Verbrennung erhielten wir im Mittel (10 Tiere) in der Leber 430,000 und in der Nierenrinde 294,000 Blutkörperchen pro mm³ Gewebe. Diagramm 1 C zeigt die Streuung der Primärwerte.

Die periphere Blutmenge ist in diesem Fall in der Leber auf das reichlich 1½fache des entsprechenden Kontrolltierwerts gestiegen, aber bezüglich der Nierenrinde lässt sich auch hier kein sicherer Einfluss konstatieren.

Um festzustellen, ob das Leberparenchym beim Brennen eine Erwärmung erfuhr, wurden während des Eingriffs Temperaturbestimmungen an verschiedenen Stellen in der Bauchhöhle vorgenommen. Die Messungen wurden mit einem empfindlichen Quecksilberthermometer ausgeführt.

Wenn die Verbrennung unterhalb des kaudalen Leberrandes liegende Hautbezirke trifft wird die Erwärmung sehr unerheblich. Nur zwischen Bauchwand und Leberparenchym, an dem verbrannten Hautgebiet unmittelbar benachbarten Stellen, liess sich eine

Temperatursteigerung von 1°C nachweisen. Tiefer in der Bauchhöhle und zwischen den Leberlappen hielt sich die Temperatur somit konstant.

Wäre der Effekt einer örtlichen Verbrennung auf die peripheren Blutgefäße der Leber eine Folge von Erwärmung des Leberparenchyms, dann würde derselbe innerhalb verschiedener Teile der Leber verschieden stark ausgeprägt sein. Ein solches Verhalten wurde indessen niemals beobachtet, weshalb der Effekt auf andere Weise zustandekommen dürfte.

Lokale Erfrierung.

An 10 Tieren wurden lokale Erfrierungen durch Bespritzen mit Chloräthyl und bei 7 Tieren mittels gekühlten Metalls, welches gegen die Haut gepresst wurde, hervorgerufen. Da die Resultate dieser beiden Gefriermethoden übereinstimmen wurden sämtliche Versuche in eine Serie zusammengefasst.

Die Mittelwerte der peripheren Blutmenge in der Leber und Nierenrinde wurden 510,000 bzw. 264,000 Blutkörperchen pro mm^3 Gewebe. Diagramm 2 D veranschaulicht die Streuung der Primärwerte.

Aus diesen Werten wird ersichtlich, dass, verglichen mit den entsprechenden Kontrolltierwerten, die periphere Blutmenge in der Leber im Mittel verdoppelt wurde, während sie in der Nierenrinde um 27 % sank.

Lokale mechanische Hautverletzung.

Die beiden Methoden zur Erzeugung einer mechanischen Hautverletzung gaben identische Resultate, weshalb die Versuche zu einer Serie vereinigt wurden. Die Mittelwerte der Bestimmungen an 9 Tieren betrugen für die Leber 359,000 und für die Nierenrinde 227,000 Blutkörperchen pro mm^3 Gewebe. Die Anordnung der Primärwerte geht aus Diagramm 2 E hervor.

Während sich also kein sicherer Effekt auf periphere Blutgefäße in der Leber konstatieren liess ist die entsprechende Blutmenge in der Nierenrinde um fast 40 % zurückgegangen.

Lokale Schädigung denervierter Haut.

Bei lokaler Verbrennung von denervierter Haut, wobei mit dem Messingblech gebrannt wurde, betrugen die Mittelwerte (11 Tiere)

für die periphere Blutmenge in der Leber 535,000 und in der Nierenrinde 273,000 Blutkörperchen pro mm³ Gewebe, gegenüber 559,000 bzw. 253,000 bei entsprechender Behandlung von Tieren mit intakter Hautinnervation. Über Streuung der Primärwerte siehe Diagramm 2 F. Die Bestimmungen der Anzahl offener Kapillaren im Masseterquerschnitt an diesen Tieren (Tab. 1) ergaben im Mittel (11 Tiere) 1,370 gegenüber 1,650 bei gebrannten Tieren mit intakter Hautinnervation. Die Differenz der mittleren Fehler bei diesen Serien beträgt 7.6, weshalb die Differenz der Mittelwerte statistisch sicher ist. Der Wert 1,370 stellt keinen statistischen Unterschied von dem Wert für die Kontrolltiere dar.

Aus diesen Werten ergibt sich, dass der Effekt auf periphere Blutgefäße in der Leber bei örtlicher Verbrennung der Bauchhaut auch dann resultiert, wenn die Haut und teilweise die Bauchmuskulatur vor dem Brennen ihrer segmentalen Innervation beraubt worden war. Dagegen bleibt der Effekt auf die Kapillaren im M. masseter in denjenigen Fällen aus, wo die verbrannte Haut denerviert war, weshalb diese Wirkung von einer intakten Hautinnervation abhängig sein muss. In keinem der Fälle wurde ein sicherer Effekt auf die entsprechenden Blutgefäße in der Nierenrinde erzielt.

Um bei diesen Versuchen mit Sicherheit ausschliessen zu können, dass der Effekt von der Innervation des geschädigten Gewebsgebiets abhängt, muss man natürlich voraussetzen, dass nicht undenervierte Gewebspartien, beispielsweise die Bauchmuskulatur (welche ja nur teilweise denerviert war), in solchem Ausmass geschädigt worden waren, dass sie auf dem Wege über die intakten Nerven auf die peripheren Blutgefäße in der Leber einwirken konnten.

Aus der obigen Beschreibung der Veränderungen bei durch Applikation des heissen Messingblechs verursachten Verbrennungen geht hervor, dass die Muskulatur nur unwesentlich geschädigt worden war. Es ist recht unwahrscheinlich, dass die begrenzten Veränderungen in der Muskulatur allein zu einer so starken nervösen Reizung führen könnten, dass die Zunahme der peripheren Blutmenge in der Leber dieselbe würde wie bei denjenigen Versuchen, wo die Innervation innerhalb des gesamten geschädigten Gewebsgebiets intakt war.

Um indessen unsere Schlussfolgerungen fernerhin zu sichern haben wir in einer Reihe von Fällen mit dem obenerwähnten

dünnen Kupferblech gebrannt, wobei pathologisch-anatomisch nur die Haut, also ausschliesslich denerviertes Gewebe, geschädigt wurde. Diese Versuche wurden an 5 Tieren mit intakter Hautinnervation und 4 Tieren, deren Haut und teilweise auch Bauchmuskulatur denerviert worden war, ausgeführt. Die Resultate sind in Tab. 2 wiedergegeben.

Tabelle 2.

Periphere Blutmenge in der Leber und Nierenrinde 45 Min. nach Erzeugung einer besonders oberflächlichen Verbrennung.

Intakte Hautinnervation				Denervierte Haut			
Nr.	Gewicht g	Anzahl Blutkörperchen in tausenden pro mm ³ Gewebe.		Nr.	Gewicht g	Anzahl Blutkörperchen in tausenden pro mm ³ Gewebe.	
		Leber	Nierenrinde			Leber	Nierenrinde
1	430	350	175	1	500	475	240
2	430	425	430	2	400	400	310
3	420	600	620	3	480	450	175
4	360	450	175	4	500	300	430
5	350	500	175				

Die in Tab. 2 angegebenen Werte für die periphere Blutmenge in der Leber zeigen im Vergleich zu den entsprechenden Kontrollwertwerten eine deutliche Zunahme, und diese Steigerung ist in beiden Versuchsreihen von ungefähr derselben Grössenordnung.

Es ist also wahrscheinlich, dass eine derartige oberflächliche Verbrennung von sowohl intakter wie denervierter Haut zu einer Zunahme der peripheren Blutmenge in der Leber führe, weshalb für wahrscheinlich gehalten werden kann, dass dieser Effekt ohne Mitwirkung der Innervation des geschädigten Gewebsgebiets zustandekommen kann.

Ein Vergleich der beiden Versuchsserien ergibt, dass der Effekt bei Verbrennung denervierter Haut etwas weniger ausgesprochen ist als bei intakter Haut, was entweder darauf zurückzuführen sein kann, dass die denervierten Tiere bei Schädigung von genau gleichgrossen Hautbezirken wie sonst grösser als die übrigen waren, oder möglicherweise ein Ausdruck dafür sein kann, dass ein Effekt auf dem Wege über die Hautnerven neben dem auf andere Weise vermittelten Effekt vorliegt.

Lokale Erfrierung von denervierter Haut wurde in 9 Fällen mit Chloräthyl und in 4 Fällen mittels gekühlten Metalls hervorgerufen. Die Mittelwerte der Bestimmungen der peripheren Blutmenge an diesen 13 Tieren wurden für die Leber 347,000 und für die Nierenrinde 276,000 Blutkörperchen pro mm³ Gewebe gegenüber 510,000 bzw. 264,000 bei der entsprechenden Behandlung von Haut mit unversehrter Innervation. Diagramm 2 G demonstriert die Streuung der Primärwerte.

Beim Vergleich der Werte für die periphere Blutmenge in der Leber und Nierenrinde bei Kälteschädigung von denervierter und intakter Haut wird ersichtlich, dass die Zunahme in der Leber im ersteren Fall nur 30 % gegenüber fast 100 % im letzteren ausmacht. Die Verringerung der peripheren Blutmenge in der Nierenrinde ist in beiden Fällen von derselben Grössenordnung.

Aus diesen Resultaten geht hervor, dass der Effekt einer lokalen Kälteschädigung auf die periphere Blutmenge in der Leber zum grössten Teil von der intakten Hautinnervation abhängig ist und somit durch die Hautnerven vermittelt werden dürfte. Ein gewisser von der Hautinnervation unabhängiger Effekt ist offenbar ausserdem wahrscheinlich. Die eventuelle Verminderung der peripheren Blutmenge in der Nierenrinde ist in beiden Fällen dieselbe, weshalb diese unabhängig von intakter Hautinnervation zustandezukommen scheint.

Erörterung der Versuchsergebnisse.

Früher scheint SIMONART (1930) der einzige gewesen zu sein, welcher das Verhalten der peripheren Blutgefässe bei der Verbrennung direkt zu studieren versuchte. Seine nichtquantitative Methodik gestattet indessen keine sicheren Schlussfolgerungen, und zwar u. a. deshalb, weil Äthernarkose, in welcher er das Brennen ausführte, an sich eine allgemeine Dilatation dieser Blutgefässe, vor allem gerade in den Bauchorganen, mitsichbringt (T. SJÖSTRAND 1935, LINDGREN 1935), weshalb es schwierig sein muss, an Hand von einzelnen Beobachtungen, um welche es sich hier handelt, zu entscheiden, ob die Dilatation eine Folge der Verbrennung oder beispielsweise einer verschiedenen Narkosetiefe bei dem gebrannten Versuchstier und dem Kontrolltier ist. Zudem sagt eine derartige Methodik nichts über die Blutfüllung der Kapillaren, sondern berücksichtigt in erster Linie die Blutfüllung in

den Arterioli und Venulae, durch welche die Farbe der Organe hauptsächlich bedingt wird.

Man kann sich vorstellen, dass die Variationen der peripheren Blutmenge in der Leber (und Nierenrinde), welche hier bei Verbrennung und Erfrierung eines begrenzten Hautbezirks nachgewiesen wurden, im Prinzip entweder durch chemische oder nervöse Vermittlung, oder durch einen direkten Temperatureinfluss auf das Parenchym der Organe zustandekommt.

Die Versuche mit Verbrennung machen offenbar wahrscheinlich, dass die Fernwirkung auf periphere Blutgefässe in der Leber bei dieser Form von Hautschädigung unabhängig von der Innervation des geschädigten Gewebsgebiets eintritt. Da zudem ausgeschlossen ist, dass der Effekt die Folge einer direkten Erwärmung des Leberparenchyms wäre, bleibt nur die Möglichkeit übrig, dass dieser Effekt *durch chemische Vermittlung* zustandegekommen ist.

Bei Verbrennungen dürften somit innerhalb des geschädigten Gewebsgebiets aktive Substanzen gebildet oder freigemacht werden können, welche nach ihrer Resorption die peripheren Blutgefässe in der Leber direkt oder indirekt beeinflussen, sodass sich dieselben dilatieren, während sie die entsprechenden Blutgefässe in der Nierenrinde entweder überhaupt unberührt lassen oder eventuell in geringerem Grade zur Kontraktion bringen. Die Muskelkapillaren, welche von diesen Substanzen nicht beeinträchtigt werden, öffnen sich in grösserem Ausmass durch eine Einwirkung auf dem Wege über die Hautnerven.

Wir stehen hier also höchst komplizierten Verhältnissen gegenüber, indem drei Organe auf ein und denselben Reiz, welcher den Organismus trifft, ganz verschieden reagieren. Es ist daher offenbar, dass Beobachtungen über Reaktionen der peripheren Blutgefässe in einem gewissen Organ bei verschiedenen Zuständen keine generellen Schlussfolgerungen gestatten.

Man kann für wahrscheinlich halten, dass der hier nachgewiesene Effekt auf die peripheren Blutgefässe mit der Ausdehnung des verbrannten Gewebsgebiets und der Dauer der schädigenden Einwirkung zunimmt, und dass die Dilatation dieser Blutgefässe bei ausgedehnten Verbrennungen einen solchen Grad erreichen kann, dass dieselbe einen mehr oder weniger bedeutungsvollen Faktor beim Zustandekommen des Verbrennungsschocks darstellen muss.

Dass es sich dabei um einen primären Faktor handeln dürfte scheint daraus hervorzugehen, dass der Effekt auf die peripheren

Blutgefäße bereits bei einer Verbrennung, welche keinen genügenden Umfang hat, um Schock herbeizuführen, und ausserdem binnen so kurzer Zeit, wie es hier der Fall ist, ein ganz ausgeprägter ist.

Vor allem scheint die Dilatation der peripheren Blutgefäße in der Leber, wenn dieselbe hochgradiger wird, einen wichtigen Schockfaktor bilden zu können. Wäre diese Annahme zutreffend, dann hätten wir wenigstens hier einen Schockfaktor, welcher mit der Intoxikationstheorie im Einklang stehen würde.

Der Effekt, welchen man durch Resorption aus dem verbrannten Hautgebiet erhält, weist keine Ähnlichkeit mit demjenigen auf, welcher durch eine Histamininjektion ausgelöst wird, was von LINDGREN (1935) studiert worden ist. Die Annahme, dass Histamin oder H-Substanz bei der Verbrennung freigemacht und resorbiert wird, findet also an dieser Untersuchung keine Stütze.

Die Resultate bei durch Erfrierung hervorgerufener örtlicher Hautschädigung weichen von denjenigen bei der Verbrennung insofern ab, als der Effekt auf die peripheren Blutgefäße in der Leber zum grössten Teil durch die Hautnerven vermittelt wird und nur ein kleiner Teil auf andere Weise. Ein Effekt durch Abkühlung des Leberparenchyms ist ausgeschlossen, da nicht einmal ein relativ anhaltender Kältereiz (siehe unsere frühere Arbeit 1940) einen derartigen Effekt herbeiführt, obwohl die Abkühlung dabei noch ausgesprochener sein muss. Derjenige Teil der Einwirkung auf die peripheren Blutgefäße in der Leber, welcher bei Erfrierung von denervierter Haut bestehenbleibt, dürfte also durch chemische Vermittlung zustandekommen.

Die eventuelle Kontraktion von peripheren Blutgefässen in der Nierenrinde ist ja von einer intakten Hautinnervation unabhängig, und es bestehen somit Möglichkeiten eines chemisch vermittelten Effekts auf diese Blutgefäße.

Versuche mit lokaler Erfrierung und Verbrennung haben ergeben, dass der Effekt auf periphere Blutgefäße in der Leber und Nierenrinde in beiden Fällen gleichartig ist; während aber die Fernwirkung bei der Verbrennung hauptsächlich oder sogar völlig durch chemische Vermittlung zustandekommt, wird dieselbe bei Erfrierung bezüglich der Leber zum grössten Teil von den Hautnerven und in geringerem Masse chemisch vermittelt.

Denjenigen Effekten von Kälteschädigung, welche HARKINS und Mitarbeiter (1934, 1935, 1937) nachgewiesen haben und die denen bei der Verbrennung ähnlich sind, können also auch die

Einwirkungen auf periphere Blutgefässe in der Leber und Nierenrinde angereicht werden, wobei hier gleichzeitig auf den möglichen Unterschied der Entstehungsweise hingewiesen werden soll.

Die Versuche mit lokaler mechanischer Hautverletzung zeigen, dass eine rein mechanische Zerfetzung der Haut nicht zu einem ähnlichen Effekt wie Verbrennung oder Erfrierung führt. Aus diesem Resultat dürfte zu entnehmen sein, dass ein blosser Schmerzreiz, welcher ja in diesen Fällen erheblich sein muss, allein während der Narkose keine beträchtlichere Dauerwirkung auf die peripheren Blutgefässe in der Leber und Nierenrinde ausüben muss.

Aus der Versuchsreihe, welche wir hiermit bis auf weiteres abgeschlossen haben, scheint hervorzugehen, dass die Hypothese, welche besagt, es werden durch verschiedene Hautreize (LAQUEUR 1930) und Schädigungen (LEWIS 1927) aus den Hautzellen Substanzen freigemacht, welche nach ihrer Resorption eine Allgemeinwirkung auf z. B. die peripheren Blutgefässe des Organismus ausüben könnten, nur hinsichtlich der Verbrennung und möglicherweise der Erfrierung eine Stütze findet. Dagegen lässt sich ein erheblicher Einfluss auf die peripheren Blutgefässe in inneren Organen bei mehreren verschiedenartigen Hautreizungen durch Vermittlung der Hautnerven erzielen.

Die Ergebnisse dürfen zu der Annahme berechtigen, dass die chemische Labilität, welche die Haut verschiedenen dieselbe treffenden Reizen gegenüber aufweist und die zu der LEWIS'schen H-Substanz-Theorie Anlass gegeben hat, lokal gebunden ist und nicht in grösserem Ausmass *chemisch* auf den übrigen Organismus einwirkt. Dagegen dürfte diese chemische Labilität dazu imstande sein, die Vorbedingungen für die Reizung der Nervenendigungen in der Haut bei z. B. Ultraviolettbestrahlung zu schaffen und auf diese Weise *auf dem Wege über die Hautnerven* innere Organe zu beeinflussen.

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Zusammenfassung.

1. Die Blutmenge in peripheren Blutgefäßen der Leber und Nierenrinde wurde 30—45 Min. nach der Erzeugung entweder einer oberflächlichen Verbrennung, Erfrierung oder mechanischen Hautverletzung in einem begrenzten Gebiet quer über der Bauchhaut an Meerschweinchen bestimmt. Bei den Versuchen mit Verbrennung wurde ausserdem die Anzahl offener Kapillaren pro Flächeneinheit des Querschnitts im M. masseter bestimmt. Die Versuche sind in Pernoctonnarkose ausgeführt worden.

2. Bei lokaler Verbrennung steigt die periphere Blutmenge in der Leber bis auf das im Mittel reichlich zweifache der entsprechenden Kontrolltierwerte, während die entsprechende Blutmenge in der Nierenrinde etwas zurückgeht oder überhaupt nicht beeinflusst wird. Die Anzahl offener Kapillaren im Querschnitt des M. masseter nimmt um 25 % zu.

3. Bei derselben Behandlung von vorher denervierten Hautbezirken wird gleichwohl dieselbe Verdopplung der peripheren Blutmenge in der Leber erzielt, sowie gleichartige Werte für die entsprechende Blutmenge in der Nierenrinde, aber keine Zunahme der Anzahl offener Kapillaren im M. masseter.

Der Effekt auf die peripheren Blutgefäße in der Leber scheint durch Resorption einer oder einiger Substanzen aus dem geschädigten Gewebsgebiet vermittelt zu werden, welche direkt oder indirekt dilatierend wirken, während der Effekt auf die Kapillaren im M. masseter auf dem Wege über die Hautnerven vermittelt wird.

4. Bei lokaler Erfrierung steigt die Blutmenge in den peripheren Blutgefäßen der Leber auf das im Mittel doppelte der Kontrolltierwerte, während die entsprechende Blutmenge in der Nierenrinde um nahezu 30 % zurückgeht.

5. Bei entsprechender Behandlung von denervierter Haut erhält man eine Zunahme der peripheren Blutmenge in der Leber von nur 30 %, während die Verminderung in der Nierenrinde dieselbe ist wie bei den sub 4 erwähnten Versuchen.

Der Effekt auf periphere Blutgefäße in der Leber scheint bei örtlicher Erfrierung also hauptsächlich auf dem Wege über die Hautnerven vermittelt zu werden, während ein kleinerer Teil der Wirkung auf periphere Blutgefäße in der Nierenrinde chemisch vermittelt werden dürfte.

6. Bei mechanischer Hautverletzung erhielt man als einzigen Effekt ein Sinken der peripheren Blutmenge in der Nierenrinde um fast 40 %.

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Electrothyreogram, Blood Iodine and Thyroid Iodine during Stimulation of the Sympathetic.¹

By

B. HELIN and H. ZILLIACUS.

In 1914 CANNON and his collaborators started a series of investigations, among which is worth mentioning elicitation of thyrotoxic symptoms by means of continuous faradic stimulation of the sympathetic in the neck or by effecting an anastomosis between the sympathetic and the phrenic nerve. In 1916 CANNON and CATTELL showed that faradic stimulation of the sympathetic or an intravenous injection of adrenaline caused an action potential in the thyroid. BURGET (1917) and MARINE and his collaborators (1918) repeated the experiments of CANNON and his collaborators, but could not produce thyrotoxic symptoms by means of stimulating the sympathetic. HANEY (1932), however, was able to establish a definitely increased metabolism by these means, a result, the correctness of which is contested by FRIEDGOOD and BEVIN (1939).

Several workers have made sections of all the nerves leading to the thyroid and then noted changes such as alterations in the normal chloride content and the normal indigo-carmin excretion. In recent years NAKAOKA (1934), PECZENIK (1935) and others have made histological observations after section or stimulation of the sympathetic. In the former case they noted atrophic glands, in the latter hyperplastic ones. ROSSINE, KRITCHEVSKAIA and SEMENOV (1936) recorded action potentials from the thyroid after stimulation of the sympathetic and after in-

¹ Received 15 October 1940.

section of adrenaline. They emphasize the dependence of the thyroid secretion on both nervous and humoral factors. HATAMA (1936) also recognizes in the action potentials, which he calls electrothyreograms, evidence of the sympathetic participating in the regulation of the secretory activity of the thyroid.

The Problem.

It has been proved anatomically as well as histologically and to some extent physiologically that many nerves, mostly of a sympathetic, but also of a parasympathetic nature, lead to the thyroid. Numerous tests have shown that these nerves affect the vasomotor processes in the gland, and many experiments tend to prove that the nerves, especially the sympathetic nerves, influence glandular secretion, but some tests have yielded negative results. A study of these facts has induced us to undertake an investigation into the correlation between action potentials (or electrothyreograms), blood iodine and thyroid iodine. Our problem is therefore: does stimulation leading to an action potential of the thyroid, also cause blood iodine and the iodine content of the thyroid to deviate from the normal in such a fashion that the electrothyreogram can be considered as an index of secretory activity in the gland? The second problem, a natural consequence of the first question, was: is the correlation between the electrothyreogram, blood iodine and thyroid iodine of such a nature as to provide a further argument in favour of the sympathetic innervation of the thyroid gland?

All electrothyreograms hitherto recorded have been elicited by sympathetic stimulation, either direct by faradic stimulation or by means of an intravenous injection of adrenaline. In no case have action potentials been noted after stimulating the vagus or its branches. In view of these circumstances we have used sympathetic stimulation in all our tests, either direct faradic excitation of the nerve or an intravenous injection of adrenaline.

The questions raised divide the experiments into the following sections:

registration of electrothyreograms during

- a) faradic stimulation of the sympathetic in the neck
- b) an intravenous injection of adrenaline

measurements of iodine content in

- a) the blood after faradic stimulation,
- b) the blood after an intravenous injection of adrenaline
- c) normal blood,
- d) normal thyroid gland substance,
- e) stimulated thyroid glands.

Electrophysiological Technique.

The action potentials were led off from decerebrate cats¹ according to descriptions by CANNON and CATTELL and by HASAMA.

The animals were operated in ether anaesthesia. All contact between tracheal cannula on the one hand, the thyroid, blood vessels and nerves in its vicinity on the other, is carefully avoided. By releasing the carotid compression during decerebration so much blood is allowed to collect in the evacuated skull that 10 ccm can be withdrawn with a syringe for the iodine analysis to be made later in the experiment, whereupon the cavity is dried and filled with cotton wool in the usual manner. After the operation the cat is left lying for two hours. Silverchloride electrodes with cotton contacts are used. The leads are taken to a balanced resistance-coupled push-pull amplifier connected to a string galvanometer. One electrode rests on the surface of the thyroid, the other is indifferent, *e. g.*, in the decerebration wound. Electronegativity in the gland corresponds to upward movement on the film. The central stump of the sympathetic is stimulated immediately below the superior cervical ganglion.

The method of recording electrothyreograms, when stimulating with adrenaline is the same as above except that now adrenaline (1 ccm 1: 70—100,000) is injected into one of the femoral veins.

Results.

a. The Electrothyreogram during Faradic Stimulation of the Sympathetic.

After compensating the demarcation current until a constant baseline is attained, the sympathetic is stimulated faradically on one side for $\frac{1}{2}$ —1 minute (distance between the coils 7—11 cm.). The gland regularly becomes electronegative in relation to the neutral point. A slow, continuous deflection of the string can be seen during which the potential difference attains its

¹ To avoid obtaining values in the subsequent iodine analyses that might be due to food rich in iodine, the test animals were placed on regular diet poor in iodine for five days before the experiments.

maximum, about 1 mV, in 2—3 minutes, thereafter to decrease continuously until the baseline is reached. The whole of this potential change lasts 5—8 minutes. With a few exceptions the gland, after stimulation, proved to be electro-negative. Frequently the curve did not return to the baseline, but remained slightly above or below the original one (Fig. 1).

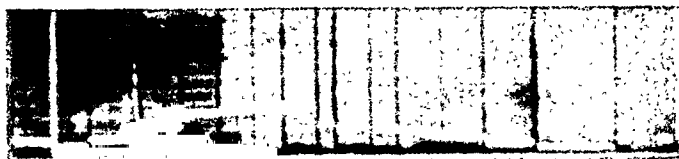


Fig. 1. Fractions of the electrothyreogram during faradic stimulation of the sympathetic. Calibration with 1 mV. Black vertical lines mark film stop.

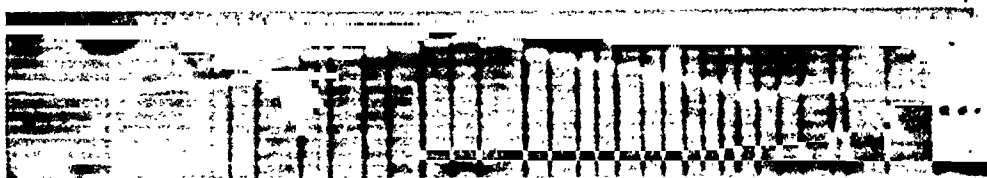


Fig. 2. The electrothyreogram during intravenous injection of adrenaline. Calibration with 1 mV.

b. The electrothyreogram during Intravenous Injection of Adrenaline.

Adrenaline that is administered intravenously (1 ccm 1: 90,000) regularly causes electro-negativity in the gland. A latent period of 5—10 seconds precedes a slow and continuously increasing potential difference which attains its maximum in about 2 minutes. The descending phase of the curve sets in just as after faradic stimulation, then proceeds continuously, and stops slightly above or below the original baseline. (Fig. 2).

The thyroid potentials after sympathetic stimulation, described above, correspond very well to those recorded by CANNON and CATTELL and by HASAMA. These authors, by means of convincing tests, came to the conclusion that the vasomotor process is not the cause of the action potentials and consider that the slow and continuous potential difference is related to the process of secretion in the gland, a statement, to which we did not wish to subscribe without further argument. A parallel

increase in blood iodine and a reduced iodine content in the thyroid gland may be regarded as such arguments.

Technique of Iodine Determination.

As already stated, the iodine determination in the blood and thyroid gland form the second part of the experiment. For this purpose we employed a modified Leipert method of micro-iodine analysis which we had subjected to special investigation. (HELIN, ZILLIACUS and UNONIUS, 1939).

a. Determination of the Iodine Content of Blood after Faradic Stimulation of the Sympathetic.

As it was a question of determining the iodine content in the blood of the test animal under the same conditions as during the recording of the electrothyreogram, faradic stimulation or adrenaline injections were carried out in exactly the same manner as before. In some cases we were even able to use the same test animals for both phases of the experiment. The general procedure has been described above: during decerebration exactly 10 ccm of blood are withdrawn with a syringe and after a couple of hours the sympathetic was stimulated (coil distance 7—10 cm) faradically immediately below the superior cervical ganglion three to five times at intervals of 40 minutes. In about half the cases the electrothyreograms were photographed. After the last period of stimulation blood samples were taken at once for two or more analyses from one of the carotids, after which the experiment was concluded by an analysis of these samples. In order to avoid disturbing prevalent conditions of equilibrium in the tissues we did not take blood samples between the periods of stimulation. (Table 1).

b. Determination of the Iodine Content in Blood after an Intravenous Injection of Adrenaline.

These tests were made under the same experimental conditions as before. The adrenaline (1 ccm 1: 70—100,000) was injected intravenously into the femoral vein and the interval between the injections was 40 minutes. During decerebration 10 ccm of blood were taken for determining normal iodine, after

Table 1.

Blood iodine content after faradic stimulation of the sympathetic.

Test animal. Cat No.	Blood iodine (γ %) before stimul.	Blood iodine (γ %) after stimulation		Faradic stimulation periods			Increase in blood iodine in %
		Indi- vidual values	Average	Num- ber	Dura- tion, min.	Strength cm	
1	23.4	27.5 30.1	28.8	3	2	10	23.1
2	33.0	33.7 33.1 34.3	33.7	4	1	12	2.1
3	24.8	27.5 28.4 29.1	28.3	4	$\frac{1}{2}$	10	14.1
4	7.4	14.2 15.4 15.3	14.9	5	1	11	101.3
5	3.7	16.1 17.3 16.9	16.7	4	1	10	351.4
6	8.1	14.5 13.5 12.4	13.5	3	1	9	66.6
7	21.1	31.5 28.5	30.0	4	1	8	42.1
Aver.	17.3		23.7	3.8	1.1	10	36.6

which the cat was allowed to lie for two hours before the first injection was made. After three to five injections accompanied by registration of gland potentials the experiment was concluded by an iodine analysis of two or more blood samples, all of which were therefore taken after the last injection had been made (Table 2).

A glance at the two tables at once shows a distinct difference between the normal blood iodine values on the one hand and the values obtained after faradic stimulation of the sympathetic or after intravenous injection of adrenaline on the other. The averages already display an increase in the blood iodine after stimulation, but, as the normal blood iodine content of the different animals varies considerably (3.7—30.0 γ %), the values for each individual animal are of greater importance. In no case it was possible to record a reduction of the blood iodine content.

Table 2.

Blood iodine content after intravenous injection of adrenalin.

Test animal Cat No.	Blood iodine (γ %) before inject.	Blood iodine (γ %) after injection		Adrenalin injections			Increase in blood iodine %
		Indi- vidual values	Average	Num- ber	Quan- tity ccm	Concentra- tion	
1	16.5	21.8 20.4	21.1	4	1	1:80000	27.8
2	6.9	19.2 19.4	19.3	3	1	1:100000	179.6
3	14.0	22.5 20.7	21.6	4	1	1:100000	54.2
4	10.2	14.6 13.3	13.6	5	1	1:100000	33.3
5	16.7	19.4 21.2 18.8	19.8	4	1	1:100000	18.6
6	7.4	11.3 10.6 9.8	10.5	4	1	1:80000	41.9
7	11.9	17.7 15.9	16.8	3	1	1:70000	41.1
Aver.	11.9		17.5	3.8	1	1:90000	47.1

c. Determination of the Iodine Content of Normal Blood.

The loss of blood during the operation and withdrawal of the first blood sample for the control causes a reduction of the blood volume, and it might therefore be assumed that the increased value obtained after stimulation was due to a mobilization of blood from a blood reservoir in the thyroid or to other changes in the internal milieu. Decerebration is also a process that might well be supposed to affect the blood iodine values. We therefore carried out a number of controls, in which, in accordance with the earlier experiments, the iodine content of the blood was determined at the time of decerebration, after which the animal was allowed to lie approximately the same time that was occupied in performing a complete stimulation test after which the iodine content of the blood was determined afresh. As the table indicates, exceedingly small variations occur in the normal blood iodine, so that it must be held certain that the

increased iodine values are due to sympathetic stimulation and not to some other circumstance in connexion with the performance of the tests. (Table 3).

Table 3.

The blood iodine content at the time of decerebration and 4—5 hours later, no stimulation of the thyroid having occurred in the interval.

Test animal Cat No.	Iodine content (γ %) of the blood at time of decerebration	Iodine content (γ %) of the blood 4—5 hours later		Blood iodine increase or decrease in %
		Individual values	Average	
1	19.6	19.8 20.4 19.3 19.9	19.9	+ 1.5
2	10.5	10.3 9.7	10.0	— 5
3	15.9	15.9 16.2 16.3	16.1	+ 1
4	22.6	22.1 22.7 22.4	22.4	— 0.9
5	14.4	13.9 15.7 14.8	14.8	— 4.1
Aver.	16.8		16.6	— 1.2

d. Determination of the Iodine Content of Normal and Stimulated Thyroid Glands.

As a further check on our results we extirpated both the thyroid lobes of each test animal and determined their iodine content separately. As the iodine content of the same gland cannot be determined both before and after stimulation and as the iodine content of a normal thyroid varies considerably in the case of each animal and even in the different lobes of the same gland, only a comparison between the average iodine content of a large number of stimulated and unstimulated glands possesses any significance. As both modes of stimulation, that we used, take effect through the sympathetic and as blood iodine

in both cases is increased in much the same manner and degree, we have not, in compiling table 4, differentiated between faradization and injection of adrenaline. In order to obtain values for unstimulated thyroids, we extirpated the thyroid glands of a dozen cats and analysed their iodine content (Table 4).

Table 4.

Iodine content in normal and sympathetically stimulated thyroid gland substance.

Iodine content in normal thyroids (% J)	Iodine content in thyroids after sym- pathetic stimulation (% J)
0.00440	0.00490
0.00217	0.00430
0.01500	0.01010
0.03370	0.00970
0.02061	0.01481
0.01574	0.01680
0.01680	0.03370
0.05508	0.02061
0.02497	0.00495
0.01259	0.00142
0.00204	0.01021
0.00318	0.00725
0.00319	0.01851
0.01112	0.01086
0.07142	0.00482
0.01510	0.00493
Average: 0.01919	0.00521
	0.00998
	0.01436
	0.01378
	0.00166
	0.00725
	Average: 0.01035

It is interesting in this connexion to mention that RAHE, ROGERS, FAWCETT and BEEBEE (1914) established a difference in the iodine content between the stimulated and unstimulated lobes of the same thyroid as early as in 1915.

According to our results, shown in table 4, the normal iodine content of a cat's thyroid gland varies a great deal and averages 0.01919 % (fresh thyroid gland substance) and after sympathetic stimulation 0.01035 %. The variations indicate that this procedure does not lend itself very well to checking our conclusions.

Discussion.

A survey of the experiments indicates that increase in the iodine content of the blood — by 36.9 % in case of electric stimulation and by 47.1 % in case of stimulation with adrenaline — means that the thyroid gland product has been transferred to the blood. And as it was possible to record an action potential from the thyroid under the same conditions of stimulation that caused the altered iodine values referred to, it must be held that the electrothyreogram and the increased blood iodine are different aspects of the same active process in the gland. This correlation scarcely leaves any room for doubt as to the electrothyreogram after sympathetic stimulation being an index of secretory activity in the thyroid. The experiments at the same time provide a further argument in favour of the thyroid gland secretion being dependent, at any rate partly, on the sympathetic nervous system.

Summary.

Electrothyreograms elicited in decerebrate cats by faradic stimulation of the sympathetic or by injection of adrenaline have been recorded with the aid of a string galvanometer and a directly-coupled balanced push-pull amplifier. The aim of this work has been to correlate blood iodine, thyroid iodine and electrothyreograms, and, if possible, to obtain evidence for sympathetic control of the thyroid gland.

Blood iodine has been determined by a modified Leipert method, described in detail in a previous communication (HELIN, ZILLIACUS, UNONIUS, 1939).

Blood iodine was found to be increased after stimulation of the sympathetic as well as after injection of adrenaline. In the former case the increase averaged 36.9 %, in the latter case 47.1 %. Controls with unstimulated animals showed that decerebration and operation around the thyroid did not as such influence the iodine concentration of the blood.

The increase in blood iodine after stimulation was found to be accompanied by a decrease of thyroid iodine amounting to about 85.4 %.

Stimulation bringing about these changes in the iodine concentration of the gland and the blood is always accompanied by an electrothyreogram which accordingly is regarded as an index of secretory activity in the gland.

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Über die Wirkung von Digitalis, Cardiazol, Coramin, Hexeton und Strychnin auf Kreislauf und Atmung des gesunden Menschen.¹

Von

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Die therapeutisch gebrauchten Kreislaufmittel üben ihre Wirkung bekanntlich in sehr verschiedener Weise aus. Manche von ihnen beeinflussen im Wesentlichen nur das Herz selbst, indem sie es zu kräftigeren Kontraktionen veranlassen. Diese Gruppe wird vor allem von den Digitalissubstanzen vertreten; nach der geläufigen Anschauung scheinen diese Stoffe nur dann eine Verbesserung des allgemeinen Kreislaufs zu bewirken, wenn das Herz insuffizient und somit ausserstande ist, das in den grossen Venen zurückströmende Blut in normalem Umfange weiterzubefördern. Andere Mittel dagegen greifen extracardial an, indem sie entweder durch periphere Wirkung auf die Gefässe oder durch Stimulierung des Vasomotorenzentrums den Gefässtonus steigern. Sie können dadurch unter Umständen das venöse Angebot an Blut vermehren und infolgedessen das Minutenvolumen des Herzens steigern. Als Beispiele für Kreislaufmittel, die peripher, an den Gefässen selbst angreifen, sind vor allem Adrenalin, Ephedrin und verschiedene Oxyephedrine zu erwähnen. Allerdings üben diese Substanzen bisweilen gleichzeitig auch eine unmittelbare Herzwirkung aus. Ausserdem bedingen sie eine Erhöhung des Stoffwechsels, wodurch ebenfalls indirekt der Kreislaufapparat zu vermehrter Arbeit angeregt wird. Die Zunahme des Minutenvolumens ist hierbei jedoch relativ grösser als die Stoffwechselsteigerung, so dass man sie nur teilweise als

¹ Der Redaktion am 15. Oktober 1940 zugegangen.

eine Folge der Stoffwechselwirkung ansehen darf. Endlich können als typische Mittel mit direkter Einwirkung auf die nervösen Zentren Cardiazol, Hexeton und Coramin, sowie Strychnin angeführt werden. Sowohl Atem- wie Vasomotorenzentrum werden hierbei primär gereizt; das Herz wird aber nur indirekt, z. B. durch Verbesserung der Blutströmung beeinflusst (vgl. HILDEBRANDT, 1937).

Für viele der erwähnten Mittel, die bei insuffizientem Kretslauf ausgedehnte Verwendung finden, sind keine oder nur spärliche quantitative Angaben bezüglich ihrer Wirkung am Menschen bekannt. Es sind zwar zahlreiche Tierversuche mitgeteilt worden, die eine günstige Kreislaufwirkung demonstrieren, es muss aber daran erinnert werden, dass in diesen Fällen so gut wie immer viel höhere relative Dosen gebraucht wurden, als sie bei der therapeutischen Behandlung kranker Menschen in Frage kommen. Ausserdem sind die Bedingungen oft vereinfacht, indem regulatorische Einrichtungen teilweise ausser Spiel gesetzt wurden. Es ist somit von praktischem Interesse, direkte Bestimmungen am Menschen auszuführen. Um eine feste Basis für Untersuchungen an Patienten mit insuffizientem Kreislauf zu schaffen, sind Versuche an gesunden Menschen wünschenswert, in der Hoffnung hierdurch zu einem besseren Verständnis des Wirkungsmechanismus zu gelangen. Für Adrenalin und verwandte Substanzen wurden solche Bestimmungen schon früher ausgeführt (v. EULER und LILJESTRAND 1927 und 1929, LILJESTRAND und LINDE 1933, BERGGREN und SÖDERBERG 1938). In der vorliegenden Mitteilung soll über entsprechende Untersuchungen mit Digitalis sowie mit den zentral angreifenden Substanzen Cardiazol, Coramin, Hexeton und Strychnin berichtet werden.

Unsere Versuche wurden an drei Studenten im Alter von 20—25 Jahren ausgeführt. Es wurden immer Standardbedingungen eingehalten. Die Versuchspersonen kamen in nüchternem Zustande ins Laboratorium, sassen in vorsätzlicher Muskelruhe etwa eine Stunde, ehe mit den Bestimmungen begonnen wurde und blieben selbstverständlich während der ganzen Versuchszeit im Zustande vollkommener Ruhe. Mundstück und Nasenklemme wurden während des ganzen Versuches nicht abgenommen. Das Mundstück war an einem Dreivegehahn befestigt und konnte durch Drehen des Hahnes entweder mit einem Lovénventil oder mit einem Gummibeutel (Fussballblase) verbunden werden. Durch das Expirationsventil wurde die Luft zu einem grossen

Tabelle

Digitotal 1 ml intravenös, entsprechend

Versuchsperson	Vor der Injektion					15—30 Min.	
	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.
A. K. . .	230	66	115/76	27.0	3.8	238	60
„ . .	232	63	113/80	21.6	3.5	233	63
D. T. . .	248	65	93/62	26.0	3.7	258	66
„ . .	220	70	—	—	2.9	221	63
H. L. . .	242	59	—	—	4.5	254	54
„ ¹ . .	276	61	114/81	20.8	4.9	264	59
	241.8 ± 8.0	64.0 ± 1.6			3.80 ± 0.31	244.7 ± 6.8	60.8 ± 1.7

¹ 1.5 ml.

Tabelle

Cardiazol 1 ml

Versuchsperson	Vor der Injektion					15—30 Min.	
	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.
A. K. . .	233	72	116/82	24.8	3.4	234	72
„ . .	236	61	112/77	26.6	4.2	254	62
D. T. . .	222	60	103/68	25.0	4.3	246	59
„ . .	231	62	100/68	23.6	4.5	226	60
H. L. . .	273	60	—	—	4.8	274	58
„ . .	246	56	—	—	3.9	251	53
„ . .	254	55	—	—	3.8	250	53
„ . .	238	56	—	—	3.5	248	56
„ . .	266	57	—	—	4.7	264	58
„ ¹	263	60	—	—	4.3	268	57
	264.2 ± 5.3	59.9 ± 1.6			4.14 ± 5.15	251.5 ± 4.7	58.8 ± 1.7

¹ 1.4 ml.

1.

0.1 g *Folia Digitalis*.

nach der Injektion			40—60 Min. nach der Injektion				
Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.
116/77	24.3	4.6	236	65	122/82	25.5	3.6
119/86	20.2	4.5	237	66	119/86	21.2	3.6
97/62	29.1	3.8	270	68	99/62	31.3	5.6
—	—	3.3	222	64	—	—	3.2
—	—	4.2	242	56	—	—	3.9
121/87	19.3	3.9	264	59	121/88	18.6	4.4
		4.05 \pm	245.2 \pm	63.0 \pm			4.03 \pm
		0.20	7.4	1.9			0.34

2.

10 % *subkutan*.

nach der Injektion			40—60 Min. nach der Injektion				
Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.
119/80	28.2	4.2	232	74	122/85	26.4	3.9
114/76	24.8	3.9	241	65	119/77	27.8	4.0
100/66	24.0	3.7	239	58	100/68	22.0	3.1
102/66	25.7	3.8	225	60	102/66	25.7	4.5
—	—	4.7	276	58	—	—	4.0
—	—	4.4	246	54	—	—	3.8
—	—	4.0	256	55	—	—	4.3
—	—	4.1	238	56	—	—	4.6
—	—	4.4	264	57	—	—	4.7
—	—	5.0	273	58	—	—	4.9
		4.22 \pm	249.0 \pm	59.5 \pm			4.18 \pm
		0.18	5.5	1.8			0.17

Tabelle
Coramin 1 ml

Versuchs- person	Vor der Injektion					15—30 Min.	
	O ₂ -Ver- brauch ml pro Min.	Pulsfre- quenz pro Min.	Blut- druck	Reduz. Ampli- tude × Puls- frequenz	Minuten- volumen des Her- zens l.	O ₂ -Ver- brauch ml pro Min.	Pulsfre- quenz pro Min.
A. K. . .	222	68	117/79	26.4	3.9	230	66
„ . .	227	66	119/86	21.2	3.3	238	64
D. T. . .	218	64	100/70	22.6	3.7	218	61
„ . .	212	68	—	—	4.8	195	69
„ . .	232	68	—	—	3.8	246	67
„ . .	219	66	—	—	3.8	221	66
H. L. . .	270	64	—	—	4.6	299	66
„ . .	258	57	—	—	3.9	253	57
	232.3 ± 7.3	65.1 ± 1.3			3.98 ± 0.17	237.5 ± 10.9	64.5 ± 1.4

Spirometer geleitet. Der Gaswechsel wurde durch Sammlung der Ausatemungsluft während 6—10 Minuten und nachheriger Gasanalyse im Haldaneapparat bestimmt. Zur Ermittlung des Herzminutenvolumens wurde durch den erwähnten Hahn die Verbindung mit dem Gummibeutel hergestellt, der vorher mit einem passenden Gemisch aus Acetylen, Sauerstoff und Stickstoff gefüllt worden war. Die Bestimmung geschah nach der Methode von GROLLMAN (1932). Die Pulsfrequenz wurde durch Palpation der Arteria radialis, der Blutdruck durch Auskultation, unter Verwendung des von LILJESTRAND und ZANDER (1928) beschriebenen Manometers festgestellt. Mit dem Produkt aus Pulsfrequenz und der sogenannten reduzierten Amplitude, d. h. des Pulsdruckes, dividiert durch den mittleren arteriellen Blutdruck, erhielten wir ein relatives Mass für die Zirkulationsgrösse (vgl. LILJESTRAND und ZANDER 1928, APÉRIA 1940).

Jeder Versuchstag wurde mit Normalbestimmungen eingeleitet. Dann wurde die zu prüfende Substanz injiziert, und weitere Bestimmungen erfolgten innerhalb der nächsten 15—30, bzw. 40—50 Minuten.

Als geeignetes Digitalispräparat benutzten wir Digitotal, das

3.

25 % subkutan.

nach der Injektion			40—60 Min. nach der Injektion				
Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude \times Pulsfrequenz	Minutenvolumen des Herzens l.
120/79	27.2	4.9	232	65	121/77	28.8	3.9
119/87	19.9	4.0	233	65	123/85	23.7	3.5
101/69	23.0	3.4	218	61	100/70	21.6	3.4
—	—	3.6	204	68	—	—	3.0
—	—	5.1	218	67	—	—	4.4
—	—	3.7	218	65	—	—	3.6
—	—	4.8	235	66	—	—	5.3
—	—	3.9	248	57	—	—	3.9
		4.18 \pm	232.4 \pm	64.3 \pm			3.88 \pm
		0.23	9.2	1.3			0.25

intravenös gegeben wurde. Von diesem Präparat entspricht 1 ml 0.1 g Digitalisblättern; die in unseren Versuchen verwendete Dosis war 1, bzw. (in einem Versuch) 1.5 ml. Die Ergebnisse sind in Tabelle 1 zusammengestellt.

Die Pulsfrequenz zeigt nach 15—30 Minuten eine statistisch nicht genügend sichergestellte Abnahme und der Blutdruck, sowohl nach 15—30, als auch besonders nach 40—60 Minuten eine unbedeutende Erhöhung, die gewöhnlich sowohl den systolischen als auch den diastolischen Druck betrifft. Das Amplitudenfrequenzprodukt bleibt aber praktisch genommen unverändert. In Übereinstimmung damit ist das Minutenvolumen des Herzens innerhalb der Fehlergrenzen konstant. Dasselbe gilt für den Sauerstoffverbrauch.

Die von STEWART (1931) mit der Methode von GROLLMAN beobachtete, schwer zu verstehende Abnahme des Minutenvolumens bei Gesunden nach Digitaliszufuhr, haben wir also nicht bestätigen können. Allerdings hat STEWART diesen Effekt erst spät (viele Stunden) nach peroraler Verabreichung gefunden; es ist aber zu erwarten, dass die Wirkung nach intravenöser Zufuhr besonders schnell und deutlich eintritt. Mit der Methode von

Tabelle

Hexeton 2 ml 10 %

Versuchs- person	Vor der Injektion					15—30 Min.	
	O ₂ -Ver- brauch ml pro Min.	Puls- frequenz pro Min.	Blut- druck	Reduz. Ampli- tude × Puls- frequenz	Minuten- volumen des Her- zens 1.	O ₂ -Ver- brauch ml pro Min.	Puls- frequenz pro Min.
A. K. . .	224	60	113/82	19.1	3.6	231	60
„ . .	222	60	108/79	18.8	3.7	246	60
D. T. . .	218	66	—	—	3.9	222	64
H. L. . .	240	56	—	—	4.5	270	55
„ . .	266	57	—	—	4.3	276	57
	234.0 ± 8.6	59.8 ± 1.7			4.00 ± 0.17	249.0 ± 10.6	59.2 ± 1.5

Tabelle

Strychninnitrat

Versuchs- person	Vor der Injektion					15—30 Min.	
	O ₂ -Ver- brauch ml pro Min.	Puls- frequenz pro Min.	Blut- druck	Reduz. Ampli- tude × Puls- frequenz	Minuten- volumen des Her- zens 1.	O ₂ -Ver- brauch ml pro Min.	Puls- frequenz pro Min.
H. L. ¹ . .	260	60	125/92	18.3	4.9	256	58
„ ² . .	248	57	119/89	16.4	4.1	245	58
„ ³ . .	228	58	116/86	20.0	3.7	223	56
	245.3	58.3			4.23	241.3	57.3

BROEMSER hat HARTL (1932) an einem Kreislaufgesunden nach intravenöser Zufuhr von 0.0005 g Strophantin eine erhebliche Abnahme des Minutenvolumens gesehen, gegen die Methode können jedoch ernste Bedenken angeführt werden (vgl. APÉRIA 1940).

¹ 1 mg Strychninnitrat subkutan.² 1.5 mg Strychninnitrat subkutan.³ 2 mg Strychninnitrat subkutan.

4.

intramuskulär.

nach der Injektion			40—60 Min. nach der Injektion				
Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.
112/81	19.8	3.5	250	64	114/82	20.9	3.4
117/89	16.4	4.8	230	64	117/81	23.2	4.1
—	—	3.7	236	67	—	—	3.3
—	—	4.4	244	57	—	—	3.9
—	—	4.9	238	56	—	—	4.5
		4.16 ± 0.25	239.6 ± 3.4	61.6 ± 2.2			3.84 ± 0.26

5.

subkutan.

nach der Injektion			40—60 Min. nach der Injektion				
Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.	O ₂ -Verbrauch ml pro Min.	Pulsfrequenz pro Min.	Blutdruck	Reduz. Amplitude × Pulsfrequenz	Minutenvolumen des Herzens l.
128/90	20.1	5.0	274	58	129/90	20.3	5.5
122/87	19.4	3.8	243	58	124/87	20.2	3.8
118/87	16.9	3.9	232	58	121/89	17.6	3.9
		4.23	249.7	58.0			4.40

Cardiazol (Pentamethylentetrazol) wurde in einer Menge von 1 ml einer 10 % Lösung subkutan injiziert; nur in einem Falle, dem letzten der Tabelle 2, wurde eine Dosis von 1.4 ml gebraucht.

Wie aus Tabelle 2 hervorgeht, weisen weder Sauerstoffverbrauch und Herzminutenvolumen, noch Pulsfrequenz oder Blutdruck irgend eine sichere Veränderung auf. Das Ergebnis steht somit im Gegensatz zu den Resultaten von HOEN und NEUTHARD (1937), die — ebenfalls nach 1 ml der 10 proz. Lösung subkutan

— eine beträchtliche Erhöhung des Minutenvolumens beobachteten. 20 Minuten nach der Injektion fanden sie eine Zunahme des Stromvolumens um 48 %. Diese Zunahme war teilweise durch Erhöhung des Stoffwechsels (um 17 %), teilweise aber durch schlechtere Ausnützung des Blutsauerstoffes bedingt. Leider wurden aber ihre Versuche nicht unter Standardbedingungen ausgeführt, was die Beweiskraft der Resultate erheblich einschränkt. Auffallend ist auch, dass 10, bzw. 30 Minuten nach der Injektion keine Zunahme, sondern sogar nach 10 Minuten eine Abnahme des Minutenvolumens von 11 % konstatiert wurde. Diese Tatsache steht in schlechter Übereinstimmung mit den Ergebnissen anderer Versuche, aus denen man schliessen kann, dass die Resorption des Cardiazols nach subkutaner Injektion rasch erfolgt.

Coramin (Pyridin- β -carbonsäurediäthylamid) wurde regelmässig in Mengen von 1 ml der 25 proz. Lösung eingespritzt. Wie Tabelle 2 zeigt, sind hier ebenfalls Stoffwechsel und Herzminutenvolumen sowie Pulsfrequenz und Blutdruck konstant geblieben. Für Coramin liegen ebenfalls Bestimmungen von HOEN und NEUTHARD vor. In ihren Versuchen war 20 Minuten nach der Injektion von 1 ml der 25 proz. Lösung der Sauerstoffverbrauch um 16 % und das Herzminutenvolumen um 127 % erhöht. Auch diese Untersuchungen wurden, ebenfalls wie die mit Cardiazol, nicht unter Einhaltung der Standardbedingungen ausgeführt. Sie zeigten regelmässig in den ersten 20 Minuten eine Steigerung des Stromvolumens, die dann aber wieder zurückging. Sehr bemerkenswert ist, dass die Pulsfrequenz etwas verlangsamt wurde, so dass das Schlagvolumen, das vor der Injektion 68 ml betrug, auf der Höhe der Wirkung den ausserordentlich hohen und unwahrscheinlichen Wert von 169 ml erreichte. In unseren Versuchen findet man keine Andeutung einer derartigen Wirkung.

Entsprechende Versuche an gesunden Menschen mit Hexeton (Methylisopropylzyklohexanon) scheinen bis jetzt nicht publiziert worden zu sein. Aus Tabelle 4 ist zu ersehen, dass 2 ml der 10 proz. Lösung, intramuskulär injiziert, ohne Wirkung bleiben. Die Verhältnisse liegen also genau wie beim Coramin.

Die Wirkung von 1—2 mg Strychninnitrat wurde an einer Versuchsperson, bei subkutaner Injektion geprüft (Tabelle 5). Auch hier ist das Ergebnis vollkommen negativ. Unter Benutzung der bereits erwähnten Broemser'schen Methode fand HARTL (1932) bei einem Patienten mit Cancer oesophagi eine Zunahme des

Minutenvolumens um 37 %, 15—30 Minuten nach der subkutanen Applikation von 1 mg Strychnin. Wir können diese Beobachtung nicht bestätigen.

Es wurde schon früher hervorgehoben, dass die stimulierende Wirkung des Cardiazols und des Coramins auf das Atemzentrum bekannt ist. Man konnte dies nicht nur in Tierversuchen, sondern auch beim Menschen, nach experimentell herabgesetzter Atmung beobachten. An gesunden Versuchspersonen, bei denen die Funktion des Atemzentrums durch eine vorherige Gabe von 0.02 g Morphin wesentlich herabgesetzt worden war, haben STEININGER und GAUBATZ (1935), sowie STANTON HICKS (1935) nach subkutaner Zufuhr von 1 ml der 10 proz. Cardiazollösung, bzw. der 25 proz. Coraminlösung eine bedeutende Steigerung der Atmung gesehen. Da wir in unseren Versuchen die Expirationsluft analysierten, also den respiratorischen Quotienten feststellten, war es uns möglich zu entscheiden, ob eine Zunahme der Atmung unter den von uns gewählten Bedingungen entstand. Trifft dies zu, so ist zu erwarten, dass eine gewisse Auswaschung von Kohlensäure stattfindet, wodurch der respiratorische Quotient erhöht werden muss. Aus Tabelle 6 geht hervor, dass keines der geprüften Mittel eine sichere Änderung des respiratorischen Quotienten bewirkt hat.

Tabelle 6.

Respiratorischer Quotient vor und nach Injektion verschiedener Stoffe.

S t o f f	Zahl der Ver- suche	Respiratorischer Quotient		
		vor der Injektion	15—30 Min. nach der Injektion	40—60 min. nach der Injektion
Cardiazol	10	0.801 \pm 0.007	0.788 \pm 0.012	0.770 \pm 0.018
Coramin	8	0.805 \pm 0.018	0.810 \pm 0.086	0.771 \pm 0.022
Hexeton	5	0.782 \pm 0.016	0.794 \pm 0.023	0.788 \pm 0.025
Strychninnitrat . . .	3	0.807	0.827	0.788
Digitotal	6	0.798 \pm 0.024	0.798 \pm 0.011	0.797 \pm 0.016

Unsere Versuche haben eindeutig ergeben, dass bei Verabreichung in den gebräuchlichen therapeutischen Dosen am gesunden Menschen, keine der von uns untersuchten Substanzen eine Einwirkung auf Kreislauf oder Atmung ausübt. Nach unserer Ansicht steht diese Tatsache in bester Übereinstimmung mit den Vor-

stellungen, die man sich über die Wirkungsweise dieser Stoffe gebildet hat. Am gesunden Menschen ist die Regulation der in Frage stehenden Funktionen so gut eingestellt, dass den gewöhnlich gebrauchten, verhältnismässig kleinen Dosen keine Wirkung zukommt. Damit soll natürlich nicht gesagt sein, dass die Verwendung derartiger kleiner Dosen bei Zuständen mit insuffizientem Kreislauf nutzlos sei. Ihre Wirkung in diesen Fällen muss aber besonders geprüft werden.

Zusammenfassung.

Therapeutische Dosen von Digitalis (Digitotal), Cardiazol, Coramin, Hexeton und Strychnin wurden gesunden Versuchspersonen unter Standardbedingungen injiziert und zeigten keine oder nur sehr schwache Wirkung auf Pulsfrequenz, Blutdruck, Herzminutenvolumen, Sauerstoffverbrauch und respiratorischen Quotienten.

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Die Wirkung des Atropins auf das Herzminutenvolumen des gesunden Menschen.¹

Von

R. DOMENJOZ.

Infolge der Schlüsselstellung seiner Angriffspunkte ist die Einwirkung des Atropins auf den Kreislaufapparat ausserordentlich komplexer Natur. Neben der Beeinflussung der Herzfrequenz und der Art des Kontraktionsablaufes, sind auch die Effekte dieser Substanz auf die Kreislauperipherie, z. B. auf die Kapillaren (v. LEDERER, 1936) für die Gesamtwirkung bedeutungsvoll. Bei der häufigen und vielfachen Anwendung des Atropins und seiner galenischen Bereitungsformen, ganz besonders auch bei Affektionen des Zirkulationssystems, überrascht es einigermassen, dass über seine Gesamtwirkung, wie sie in integralhafter Weise im Herzminutenvolumen zum Ausdruck gelangt, keine einheitlichen Vorstellungen herrschen.

Die Veränderungen der Förderleistung des Herzens unter dem Einfluss von Atropin sind schon öfters Gegenstand experimenteller Untersuchungen gewesen. Zum ersten mal wurde eine Steigerung des Minutenvolumens von ODAIRA (1925) beobachtet. In Versuchen an Kaninchen mit der FICK'schen Methode konnte dieser Autor nach intravenöser Verabreichung von Atropinsulfat in Mengen von 0.2 bis 0.55 mg pro kg Tier, eine Zunahme der Herzleistung zwischen 4 und 33 % konstatieren. Diese Feststellungen konnten jedoch von anderen Untersuchern nicht bestätigt werden. MARSHALL (1926), der ebenfalls mit der FICK'schen Methode die Korrelationen zwischen Herzfrequenz und Schlagvolumen studierte, konnte bei Gaben von 3—4 mg keine Zunahme des Minuten-

¹ Eingegangen am 1. November 1940.

volumens registrieren. MARSHALL verwendete zu seinen Versuchen trainierte Hunde, die eine Durchführung der Untersuchungen ohne Narkose ermöglichten. Auch HARRISON, BLALOCK, PILCHER und WILSON (1927) sahen bei Steigerungen der Pulsfrequenz zwischen 100 und 400 % des Ausgangswertes nur Änderungen in der Förderleistung des Herzens, die sich innerhalb der Fehlergrenzen der verwendeten Methode bewegten. Im Mittel fanden sie, sowohl an morphinisierten, als auch an unnarkotisierten Hunden eine Zunahme des Minutenvolumens von 4 %.

Prinzipiell ist zu diesen Tierversuchen zu sagen, dass die Genauigkeit der damit erreichten Resultate nur gering ist, sodass unbedeutende Steigerungen des Minutenvolumens wegen der weiten Fehlergrenzen statistisch nicht einwandfrei nachgewiesen werden können. Der Grund hierfür liegt wohl darin, dass die Standardbedingungen, zumal bei unnarkotisierten Tieren, nur sehr unvollkommen eingehalten werden können. Weiterhin ist die Bedeutung der Reaktionslage für die Wirkung vegetativer Gifte von so ausschlaggebender Bedeutung, dass eine Übertragung der an Tieren gewonnenen Resultate auf die Verhältnisse am Menschen nur mit Vorbehalt möglich ist.

Die Frage der Einwirkung des Atropins auf das Herzminutenvolumen des Menschen erfuhr eine erstmalige Bearbeitung durch SMITH, BURWELL und DE VITO (1928). Das hierbei verwendete Material bestand aus gesunden Studenten, an denen das Minutenvolumen nach der Methode von FIELD und BOCK bestimmt wurde. Atropin wurde intravenös, in Mengen von 1.2 mg verabreicht. Die in diesen Versuchen beobachteten Steigerungen der Pulsfrequenz gehen bis zu 40 % und die Zunahme des Minutenvolumens bis zu 10 % der Ausgangswerte.

Methodik.

Unsere Versuche wurden an 2 gesunden Erwachsenen, in nüchternem Zustande, bei vollkommener, willkürlicher Muskelruhe durchgeführt. Zu Beginn jeder Untersuchung wurde eine Ruheperiode von ungefähr 1 Stunde eingeschaltet, während der die Versuchsperson gut bedeckt in einem Liegestuhl sass. Darauf wurde mit Bestimmungen der Ruhewerte in folgender Reihenfolge begonnen: Pulsfrequenz, Blutdruck, Sauerstoffverbrauch und Herzminutenvolumen. In jedem Versuch wurden 2 Normalbestimmungen durchgeführt. Atropinsulfat wurde in einer Menge

von 0.001 g subkutan injiziert und die Wirkung an Hand der Pulsfrequenz verfolgt. Die weiteren Bestimmungen geschahen in wechselnden Intervallen, 30 bis 90 Minuten nach der Injektion.

Die Versuchsperson atmete mittels eines Kautschukmundstückes durch einen weiten Dreiwegehahn, der die Verbindung mit einem Spirometer oder mit einem Gummibeutel ermöglichte, der das Acetylgemisch enthielt.

Die Blutdruckmessung erfolgte nach der auskultatorischen Methode mit Hilfe des von LILJESTRAND und ZANDER (1928) beschriebenen Methylenjodidmanometers.

Zur Ermittlung des Sauerstoffverbrauches wurde die Expirationsluft während 7 bis 8 Minuten in einem grossen Spirometer gesammelt und die Ventilationsgrösse pro Zeiteinheit bestimmt.

Die Bestimmung des Minutenvolumens erfolgte nach der Acetylenmethode von GROLLMAN (1932). Das von uns verwendete Gasgemisch hatte folgende Zusammensetzung: 7 L Luft, 3 L Narcylen und 0.5 L Sauerstoff. Die Entnahme der Proben geschah nach 5—6, bzw. nach 8—9, tiefen, rasch aufeinanderfolgenden Atemzügen, entsprechend einer Zeitspanne von 18—20, bzw. von 25—30 Sekunden.

Neben dem Minutenvolumen wurde zur Abschätzung der Herzleistung noch das sogen. »reduzierte Amplitudenfrequenzprodukt« nach LILJESTRAND und ZANDER (1928) bestimmt. Diese Grösse, in den Tabellen als A. F. P. vermerkt, ergibt sich als Produkt von Pulsfrequenz und sogen. reduzierter Amplitude, wobei unter reduzierter Amplitude das Verhältnis des Pulsdruckes (Amplitude) zum Mitteldruck (arithmetisches Mittel aus systolischem und diastolischem Druck) zu verstehen ist.

Resultate.

Die in unseren Versuchen gefundene Steigerung des Herzminutenvolumens erscheint gering, wenn man sie mit Werten vergleicht, wie sie z. B. bei Verabreichung von Adrenalin und seiner Verwandten (von EULER und LILJESTRAND (1927 und 1929), LILJESTRAND und LINDE (1933)) oder gar in Arbeitsversuchen zutage treten. Für die Beweiskraft derartiger Untersuchungen ergibt sich hieraus, dass das peinliche Einhalten der Standardbedingungen während der ganzen Versuchsperiode von ausschlaggebender Bedeutung ist. Wir haben diesem Faktor ganz besondere Bedeutung geschenkt. In jedem Versuch wurden, nach einer ausgiebigen

Tabelle

Veränderungen von Blutdruck, Pulsfrequenz, Sauerstoffverbrauch,
0.001 g Atropinsulfat.

N o r m a l w e r t e							
Datum	Blut- druck	Puls- frequ.	Mittel- druck	A. F. P.	Sauer- stoff- verbr.	Art.- venöse Diff.	Min.- Vol.
15. X.	115/82	55	98.5	18.4	224	58.1	3.9
	115/83	55	99	17.8	224	57.4	3.9
17. X.	119/88	56	103.5	16.8	216	52.8	4.1
	123/94	56	108.5	15.0	218	52.7	4.1
19. X.	119/89	57	104	16.5	208	52.8	4.0
	119/89	57	104	16.5	215	52.7	4.1
Mittelwerte:					217.5		4.0
Streuung:					± 6.1		± 0.1

Tabelle

Veränderungen von Blutdruck, Pulsfrequenz, Sauerstoffverbrauch,
0,001 g Atropinsulfat.

N o r m a l w e r t e							
Datum	Blut- druck	Puls- frequ.	Mittel- druck	A. F. P.	Sauer- stoff- verbr.	Art.- venöse Diff.	Min.- Vol.
21. X.	109/76	58	92.5	20.7	211	57.1	3.7
	120/88	52	104	16.0	212	59.8	3.5
23. X.	105/75	58	90	19.8	205	56.8	3.6
	114/84	58	99	17.6	205	59.5	3.4
25. X.	110/78	62	94	21.1	207	55.0	3.8
	120/82	58	101	21.8	197	57.4	3.4
Mittelwerte;					206.0		3.6
Streuung:					± 5.5		± 0.17

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Rate of Penetration of Ions through the Capillary Wall.¹

By

L. HAHN and G. HEVESY.

(With 3 figures in the text.)

In this paper, the results of experiments are communicated which were carried out in order to get information on the rate of passage of the ions of important constituents of the plasma as sodium, potassium, chlorine, and phosphate through the capillary wall. Crystalline substances introduced into the circulation will soon invade the extracellular fluid of the body. On this fact is based the method usually applied to determine the size of the extracellular space. Sucrose, sulphocyanate, or sulphate introduced into the human circulation were found (LAVIETES et alia 1936), for example, to be completely distributed between the plasma and the tissue space in the course of two or three hours. A complete distribution of thiocyanate in the extracellular space of rabbits in the course of half an hour is recorded (KROGH 1937).

The partition of a substance introduced into the circulation between plasma and the extracellular fluid involves two processes: (1) penetration across the capillary wall and (2) distribution by diffusion and convective processes in the capillary and the extracellular fluids. The last mentioned processes will play a secondary role, only, in view of the very short distances between the capillaries. Taking the length of the distances involved (KROGH 1926) to be less than $60\ \mu$ and the diffusion coefficient of the substance investigated to be at least $1\ \text{cm}^2$ per day, the time necessary

¹ Received 11 November 1940.

to displace, for example, a sodium ion from one end of the capillary space to the other, or from one end of the corresponding extracellular space to the other, will be less than 2 sec.¹ We arrive at this result by considering the propagation by diffusion only of the substance which penetrated the capillary wall. The fluid is, however, not without a circulation of its own, and this circulation will possibly shorten the time arrived at in the above calculation.

By introducing some sodium chloride into the circulation and by measuring the time it takes for a certain fraction to leave the circulation it should be possible to measure the rate of passage of sodium chloride through the endothelium. However, when carrying out these experiments we meet the following difficulties: (a) Not only does the circulation get rid of excess sodium chloride by giving off salt to the extracellular space, but also by taking water up from the tissues. KEYS (1937) found, when studying the fate of sucrose intravenously injected into man, that osmotic equilibrium by a shift of water takes place from three to ten times as fast as sucrose exchange. The rate of disappearance of the excess sodium chloride will, thus, not measure the rate of passage of sodium chloride through the capillary wall but a more complex process. (b) We do not measure by the method outlined the rate of passage of sodium through the endothelium but a resultant of the rate of passage of sodium and chloride. The resistance of the endothelium to the passage of sodium and chloride may be quite different. (c) The introduction of appreciable amounts of sodium chloride into the circulation will disturb the normal conditions prevailing in the circulation. When one tries to eliminate this difficulty by introducing small amounts only, the analytical difficulties become almost unsurmountable. All these difficulties can be eliminated by injecting into the veins labelled sodium chloride (sodium chloride containing some radioactive ^{24}Na of negligible weight) and by measuring the rate of disappearance of the active ions from the plasma, i. e. the decrease of the radioactivity of the plasma. We are not determining in these experiments the rate of influx of excess sodium chloride from the plasma into the extracellular fluid but the rate of exchange between labelled plasma sodium and non-labelled extracellular sodium, as the number of sodium ($^{23}\text{Na} + ^{24}\text{Na}$) atoms of the plasma remains

¹ The mean displacement of a particle $\tau = \sqrt{2D}$, where D is the diffusion coefficient.

practically constant all through the experiment. The rate of exchange will be determined by the permeability of the capillary wall to sodium ions and will, thus, be a measure of this permeability.

We carried out also experiments with radio-potassium, radio-chlorine, radio-bromine, and radio-phosphate, while heavy water was used as an indicator for water in the study of permeability of the endothelium to water. The measurement of the distribution of radio-sodium between plasma and body of the rabbit was previously used to determine the extracellular volume of the rabbit (GRIFFITH and MARGRAITH 1939; HAHN et alia 1939).

Experimental Procedure.

Radioactive sodium and potassium were prepared by bombarding NaOH and KOH, respectively, with high speed (10 million volts) deuterons. The hydroxydes were neutralized with hydrochloric acid and the solution thus obtained was injected. Radioactive chlorine and bromine were prepared by bombarding NaCl and NaBr, respectively, with deuterons. The active chlorine and bromine obtained were driven off as HCl and HBr, respectively, and were collected in a sodium hydroxyde solution. This procedure was chosen to get rid of the active sodium simultaneously produced with the active halogens. We are much indebted to Dr. J. C. JACOBSEN and Mr. O. N. LASSEN for preparing the radioactive substances by making use of the Copenhagen cyclotron.

About 3 cc. solution containing the radioactive substances of an activity of about 1 microCurie was applied. The salt concentration of these solutions was brought up to a physiological level by adding non-active sodium chloride. The solution was injected into the jugularis of the rabbit and blood samples of about 1 cc. were collected at intervals from the carotis. Plasma samples of known weight were dried and their radioactivity was compared by using a Geiger counter. For comparison of the radiocativity of plasma and muscle samples the samples were ashed at about 400° and the plasma ash mixed with non-active muscle ash of the same weight as the corresponding active muscle ash sample. Blood and muscle samples were secured simultaneously from the narcotized rabbit.

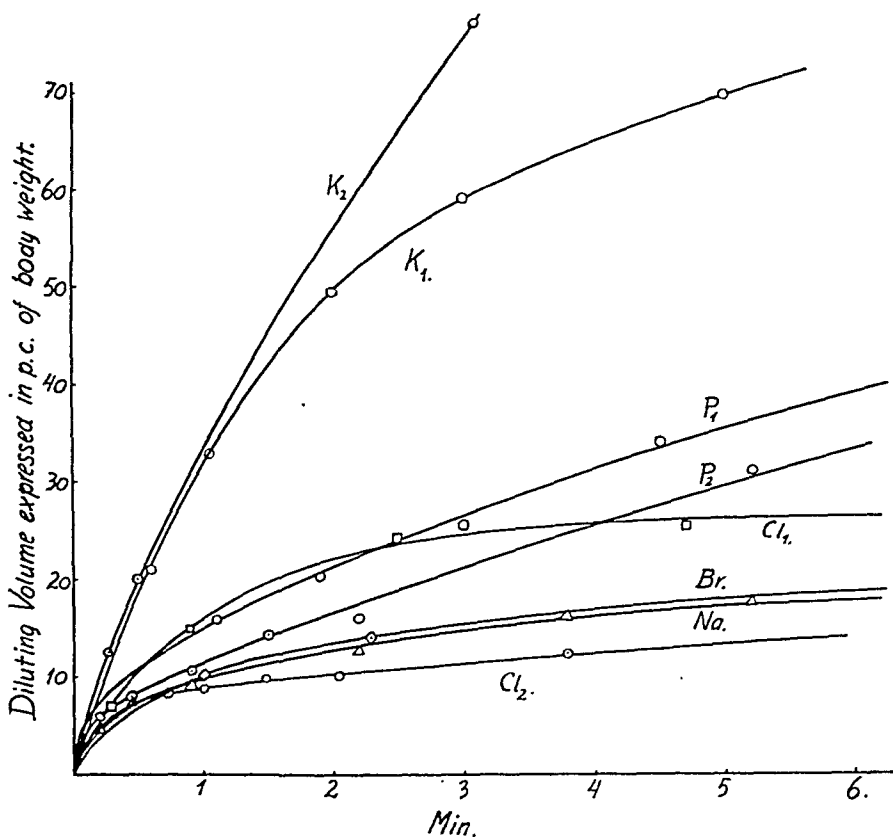


Fig. I. Rate of disappearance of various labelled ions from the plasma.

Results.

The results obtained are seen in Tables 1 to 5 and Figs. I to III. The tables contain data on the percentage of the labelled element injected still present in 1 cc. plasma at various intervals. The volume of diluting fluid necessary to bring down the concentration of the substance injected to that found after a given time is also stated. Furthermore, this diluting volume is expressed in percent of the rabbit's body weight.

We shall first compare the rate of disappearance of sodium, chlorine, and bromine from the circulation. This comparison encounters no difficulties since practically the sole outlet¹ of these elements from the circulation is the extracellular body fluid, though some ²⁴Na is taken up by the surface layer of the bone

¹ In experiments taking up to 1 hour, the amount of ²⁴Na lost by excretion is much less than 1 per cent of the amount administered.

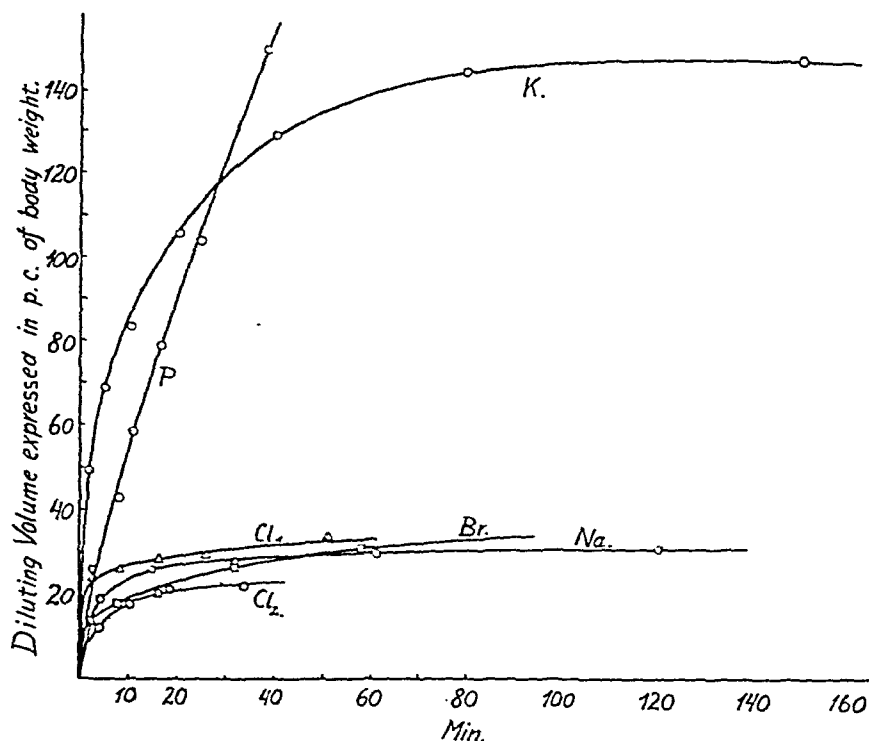


Fig. II. Rate of disappearance of various labelled ions from the plasma.

apatite (HAHN et alia 1939). No great difference is found between the rate of passage of sodium, chlorine, and bromine through the capillary wall and the values obtained for different rabbits show fairly large variations. These variations are to some extent due to differences in the size of the extracellular space which is found by numerous experimenters to show quite appreciable differences for different rabbits.

A comparison of the rate of passage of potassium, phosphate, and water with that of sodium, chlorine, and bromine encounters some difficulties since potassium, and the same applies to phosphate and water, has an additional outlet into the tissue cells in contrast to the first mentioned group. The amount of ^{42}K lost by the blood after the lapse of a given time is the resultant of the amount penetrated into the tissue fluids and that returned from the latter into the blood. When besides the interspaces the cellular space opens an outlet to the ^{42}K leaving the circulation, the amount returning from the tissue fluids into the blood will be reduced and, thus, the resultant ^{42}K concentration of the plasma will be lowered. Though the potassium content of the cells is

Table 1.

Rate of disappearance of ^{38}Cl from the circulation of rabbits weighing 2.5 and 2.4 kg, respectively.

Time in min.	Percent of ^{38}Cl injected present in 1 gm. plasma	Diluting fluid volume	
		in cc. (apparent extracellular volume)	in percent of body weight
<i>Rabbit I.</i>			
0.37	0.622	161	6.4
0.73	0.486	206	8.2
1.01	0.475	211	8.5
1.48	0.408	245	9.8
2.05	0.400	250	10.0
3.8	0.329	304	12.2
10.5	0.224	446	17.8
18.5	0.188	532	21.3
35	0.182	550	22.0
<i>Rabbit II.</i>			
0.8	0.62	161	6.7
0.9	0.28	357	14.9
2.5	0.174	575	24.0
4.7	0.165	607	25.3
8.3	0.161	622	26.0
16.5	0.150	668	27.8
26	0.143	700	29.2
51	0.128	783	32.6

only partly replaced by ^{42}K during the experiment (HAHN et alia 1939) in view of the low potassium content of the plasma and the high content of the tissue cells the additional outlet opened by the intrusion of ^{42}K into the cells in experiments taking one hour makes out about five times the normal outlet of intrusion of these ions into the interspaces.

The total water content of the cells can be entirely replaced by labelled water. Since the volume of the cellular body water is about twice as large as that of the extracellular fluid through the intrusion of labelled water into the cells a substantial additional outlet of the labelled water molecules of the plasma is opened.

Table 2.

Rate of the disappearance of ^{80}Br from the circulation of a rabbit weighing 2.7 kg.

Time in min.	Percent of ^{80}Br injected present in 1 gm. plasma	Diluting fluid volume	
		in cc. (apparent extracellular volume)	in percent of body weight
1.0	0.37	270	10.0
2.2	0.27	370	13.7
8.1	0.21	475	17.6
16.3	0.18	556	20.6
32	0.14	715	26.5
58.5	0.12	835	30.9

Table 3.

Rate of disappearance of ^{24}Na from the circulation of rabbits weighing 2.7 kg. and 2.4 kg., respectively.

Time in min.	Percent of ^{24}Na injected present in 1 gm. plasma	Diluting fluid volume	
		in cc. (apparent extracellular volume)	in percent of body weight
<i>Rab it I.</i>			
0.2	0.80	125	4.6
0.45	0.50	199	7.4
0.9	0.41	242	9.0
1.5	0.32	310	11.5
2.2	0.30	331	12.3
3.8	0.234	427	15.8
5.2	0.215	466	17.3
11	0.194	515	19.1
<i>Rabbit II.</i>			
1.8	0.63	158	6.6
2.3	0.282	357	14.9
4.3	0.218	458	19.1
9.1	0.181	553	23.0
15	0.163	615	25.6
32	0.151	663	27.6
61	0.140	715	29.8
120	0.134	745	31.1

Table 4.

Rate of disappearance of ^{42}K from the circulation of rabbits weighing 2.5, 2.4 and 2.3 kg, respectively.

Time in min.	Percent of ^{42}K injected present in 1 gm. plasma	Diluting fluid volume	
		in cc. (apparent extracellular volume)	in percent of body weight
<i>Rabbit I.</i>			
0.6	0.19	526	21
2.0	0.082	1220	49
3	0.068	1470	59
5	0.058	1720	69
15	0.036	2860	114
<i>Rabbit II.</i>			
5	0.058	1730	72
10.5	0.048	2080	87
20.5	0.038	2640	110
40.5	0.031	3220	134
80	0.0277	3610	151
210	0.0269	3720	155
<i>Rabbit III.</i>			
0.26	0.354	283	12.3
0.50	0.220	455	19.8
1.05	0.129	775	33.7
3.05	0.057	1755	76.3

As to the phosphate ions, not only that they diffuse into the tissue cells but they are also incorporated into the surface layer of the bone apatite. These additional outlets may be made responsible for the fact that the ^{32}P loss of the plasma is found to be very much greater than the ^{24}Na loss during the same time.

While the additional outlets mentioned above for potassium, phosphate, and water will be responsible for the high values of the volume of the diluting body fluid observed for these elements in experiments of comparatively long duration, the fact that also after the lapse of $\frac{1}{4}$ minute only, larger amounts of potassium than of sodium, chlorine, or bromine are lost by the plasma requires another explanation. After such a short time the volume

Table 5.

Rate of disappearance of ^{32}P from the circulation of rabbits weighing 2.1 and 2.7 kg., respectively.

Time in min.	Percent of ^{32}P injected present in 1 gm. plasma	Diluting fluid volume	
		in cc. (apparent extracellular volume)	in percent of body weight
<i>Rabbit I.</i>			
1.1	0.300	333	15.9
1.9	0.234	427	20.4
3.0	0.187	535	25.5
4.5	0.143	699	33.3
6.8	0.112	892	42.5
10.9	0.081	1230	58.6
16.9	0.060	1670	79.5
25.9	0.046	2180	104
39.0	0.032	3130	149
<i>Rabbit II.</i>			
0.2	0.63	160	5.9
0.45	0.46	216	8.0
0.9	0.35	287	10.6
1.5	0.261	383	14.2
2.2	0.234	428	15.9
5.2	0.120	835	30.9

of the diluting fluid is much smaller than the extracellular space of the rabbit and the additional outlet can, therefore, not play any decisive role. The very rapid disappearance of potassium from the circulation suggests the assumption that potassium, when passing the endothelium, encounters appreciably less resistance than does sodium or chlorine. The diffusion constant of potassium in water is larger than that of sodium; taking the former to be 1, the diffusion constant of sodium makes out 0.65. The diffusion constants of potassium and chlorine are practically identical. The rates of penetration of potassium and chlorine through the endothelium, however, differ greatly. The diffusion rate for water in water was found, using heavy water as an indicator, to be 1.6 times larger, only, than that of chlorine or potassium, while the rate of passage of water through the endothelium is very much

faster than that of any other substance investigated by us. In the course of 21 sec. the labelled water introduced into the circulation of the rabbit is found to be distributed in 506 cc. body water, corresponding to 34 percent of the rabbit's weight.

When investigating the staining capacity of dyes it was found that generally the rate of coloration increases with decreasing diffusion rate in water (ROUS et alia 1930; SMITH and ROUS 1931; MENKIN and MENKIN 1930). From the capillaries of the frogs mesentery trypan blue was found to disappear exponentially with a half-time period of 2 min. (MENKIN and MENKIN 1939).

We have not yet mentioned the fact that an outlet of the plasma ^{24}Na , for example, is given by intrusion into the corpuscles. This outlet is a very restricted one. We found the ^{24}Na content of 1 gm. corpuscles of the rabbit to be, after the lapse of two hours, 11 percent of that of 1 gm. plasma. From this figure and the haematocrit value (34 percent) of rabbits blood it follows that, expressed in diluting body fluid volume, the uptake of ^{24}Na by the corpuscles corresponds to somewhat less than 4 cc., while the total diluting volume of a rabbit weighing 2.5 kg. makes out as much as about 700 cc. After the lapse of $1\frac{1}{2}$ hours, the ^{42}K content of 1 gm. corpuscles was found to be 40 percent of that of 1 gm. plasma and the ^{32}P content 48 percent. These figures correspond to an additional diluting volume of 14 and 16 cc., respectively. The water content of the corpuscles of the rabbit being about 63 percent, the role of the corpuscles as additional outlet of the labelled water molecules introduced into the plasma is not quite insignificant.

Permeability of Muscle and Brain Capillaries.

The figures stated in the preceding section give information on the rate at which ions and molecules leave the capillary system. Should a minor part of the capillary wall be slightly permeable or even impermeable to some of the substances investigated, this would not have been revealed by the figures given above, since these figures indicate the permeability of the very inhomogeneous capillary system *in toto*. If we want to know the average permeability of the muscle capillaries, for example, to ^{24}Na , we have to compare the ^{24}Na content of plasma and muscle samples of known weight.

The results of such measurements are seen in Table 6, in which the percentage ratio of the ^{24}Na content of 1 gm. fresh gastrocne-

Table 6.

Ratio of the ^{24}Na and ^{32}P content, respectively, of 1 gm. tissue and 1 gm. plasma.

Tissue	Time in min.	Ratio of the content of 1 gm. tissue and 1 gm. plasma $\times 100$	
		^{24}Na	^{32}P
Muscle	0.9	3.74	0.98
Muscle	5.2	7.75	3.42
Muscle	11	10.7	8.82
Muscle (other rabbit)	120	11.5	—
Brain, total	11	3.0	4.5
Brain, white	11	2.2	—
Brain, grey	11	3.9	—
Brain, white (other rabbit)	120	10.9	—
Brain, grey (other rabbit)	120	14.9	—
Medulla oblongata (other rabbit) . .	120	17.2	—

mius tissue to 1 gm. plasma is given. The table contains also data on the ^{32}P content of muscles and the ^{24}Na and ^{32}P content of the brain tissue. After the lapse of 11 min., a proportional partition of ^{24}Na between plasma and gastrocnemius is nearly reached, since the size of the extracellular space of the gastrocnemius of the rabbit is about 11 per cent of the weight of the muscles. The muscle capillaries are seen to be more permeable to sodium than to phosphate: in the course of the first minute about four times more ^{24}Na left the plasma for the muscle tissue than ^{32}P .

The permeability of the brain capillaries to ^{24}Na and also to ^{32}P is lower than that of the muscle capillaries, those of the white brain substance being apparently less permeable than those of the grey brain substance to ^{24}Na .¹ After the lapse of 62 hours, we found (HAHN et alia 1939) the ^{24}Na content of 1 gm. brain to make out 32 percent of that of 1 gm. plasma, a figure which corresponds to that stated (MANERY and HASTINGS 1939) for the sodium space of the brain.

MANERY and BALE (1939) carried out experiments with labelled sodium and phosphorus. They state that, in the course of an hour,

¹ This is probably an expression of the fact that the vascularity of the grey matter greatly exceeds that of the white.

the ^{24}Na uptake by the brain and the sciatic new is much smaller than to be expected in the case of a proportional partition of ^{24}Na between plasma and interspaces of these organs while, in the case of the other organs, such a partition was reached after the lapse of 20 min. WALLACE and BRODIE (1937, 1939) investigated the distribution of iodide, thiocyanate and chloride in various tissues of the body and found that the relative concentration in terms of blood tissue ratio was alike for the three substances in the organs examined, with the exception of the brain in which the chloride was in much larger amounts than the other two. In a later investigation (1939) these authors found that these anions distribute in the central nervous system in the same ratio to chloride as in spinal fluid, whereas in other body tissues they distribute in the same ratio to chloride as in serum. That sulphate passes more slowly than bromide or nitrate through the brain capillaries can be concluded from experiments in which the chloride content of the plasma was replaced by sulphate (AMBERSON *et alia* 1938), bromide (WEIR 1936), and nitrate (HIALT 1939), respectively.

While the capillary wall in toto was found to be more permeable to phosphate ions than to sodium ions the opposite is the case for the wall of muscle capillaries. This fact suggests that a substantial part of the capillary system, possibly that of the bone, must be very easily permeable to phosphate.

We compared, furthermore, the activity of 1 gm. of the grey brain substance with that of 1 gm. plasma 59 min. after the administration of radiobromide. The ratio found was 9.3 per cent. As the chlorine space of the brain tissue of the rabbit is 35 per cent, we have to follow that in the course of an hour, less than $\frac{1}{3}$ of the proportional partition of bromine between plasma and the extracellular volume of the brain is obtained.

Permeability to Water.

We investigated previously the rate at which heavy water introduced into the circulation leaves the plasma (HEVESY and JACOBSEN 1940). We extended these measurements by determining the distribution of labelled water between plasma and gastrocnemius of equal weight. The figures obtained are seen in Table 7. The water content of the samples was driven off and was collected in vacuo as described previously. We are much indebted to Miss HILDE LEVI for kindly determining the density

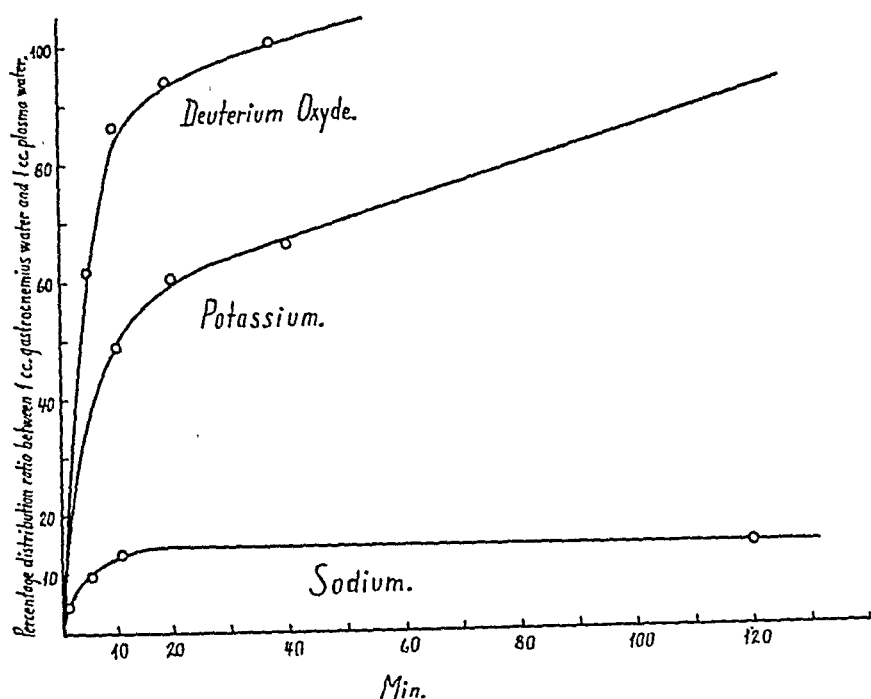


Fig. III. Percentage distribution ratio of labelled sodium, potassium and deuterium oxide between plasma water and muscle water of equal weight.

Table 7.

Ratio of the density excess of plasma water and muscle water.

Time in min.	Ratio of density excess of plasma water and muscle water
5	1.62
10	1.16
20	1.06
38	1.00

of the water samples obtained by making use of LINDERSTRØM-LANG's floating drop method.

The density excess of these water samples over the density of normal body water is a measure of their labelled water content. The ratio of the density excess of plasma water and muscle water is stated in Table 7 and Figure III.

A ratio equal to 1 is to be expected when the heavy water concentration of the total muscle water corresponds to that of the plasma water. This stage of partition is reached between 20 and 38 min. after the start of the experiment.¹ This is the same result at which HEVESY and JACOBSEN (1940) arrived when investigating the rate of disappearance of heavy water from the circulation. The water content of the gastrocnemius makes out 77 per cent of the muscles weight, about 11 per cent being located to the inter-spaces and the rest to the cells. If the extracellular water would alone take part in the exchange process, we should expect in the case of a proportional partition of the heavy water between plasma and extracellular water the ratio of the density excess to be about 7. From the fact that this ratio is found after the lapse of 5 min. to be but 1.62 we have to conclude that during that time not only a proportional partition of the labelled water between plasma and the extracellular fluid of the muscles took place but a large part of the cell water was replaced by plasma water as well.

Summary.

Solutions of labelled chloride, bromide, sodium, potassium, phosphate and deuterium oxyde were injected into the circulation of rabbits and the speed of the escape of the labelled ions from the plasma was determined. Potassium was found to penetrate at a much faster rate through the capillary wall than any other ion investigated.

Information on the permeability of the muscle and brain capillaries were obtained by comparing the labelled sodium, phosphate and heavy water content of muscle and plasma, respectively brain and plasma. The muscle endothelium was found to be more permeable to sodium and to phosphate than the endothelium of the brain. The partition ratio of labelled sodium between plasma and the chloride space of the brain amounts after the lapse of 11 min. only to $\frac{1}{10}$ of the equilibrium value; during that time a proportional partition of labelled sodium between plasma and the chloride space of the muscle was obtained.

In the course of an hour, somewhat less than $\frac{1}{3}$ of the proportional partition of bromine between plasma and the grey brain

¹ Though great care was taken to distill off the total water content of the muscle, the possibility that a minor amount of muscle water was not removed can not be excluded. This water may not have taken part in an exchange process.

substance was reached. Proportional partition of labelled water between plasma and the muscle water was reached after about half an hour.

We wish to express our hearty thanks to Professor NIELS BOHR for kindly putting numerous facilities at our disposal.

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Studies on the Muscular Physiology of the Genital Tract.

II. Tonus, Spontaneous Activity and Drug Reactions in the Cervical Muscles.¹

By

SUNE GENELL.

(With 6 figures in the text.)

In the extensive research-work devoted to the physiology of the uterine muscles (review in GENELL, 1940) almost no attention has been given to the cervical muscles. Yet the anatomical situation of the cervix as an intermediate link between vagina and uterus is in itself a reason for assuming that its muscular apparatus has an important function. The present investigation has been carried out on *rats*.

Tonus.

In a series of experiments for another purpose (GENELL, 1939) the observation was made that an injection-needle furnished with a 1-mm broad bulb passes through the cervical canal of a non-narcotized animal with ease when the animal is in dioestrus, but with difficulty when it is in oestrus. This observation suggested the thought that the tonus conditions in the cervical musculature differed from those in the rest of the uterine muscles.

Method.

The calibre of the cervical canal was measured by means of a series of measuring-cylinders resembling miniature Hegar dilators and graduated in sizes of 0.5, 0.8, 1, 1.5, 2, 2.5, and 3 mm. An assistant held the non-narcotized animal. As vaginal speculum an

¹ Received 15 November 1940.

ordinary funnel-shaped ear speculum of suitable size was used. A head-lamp or head-mirror facilitates the work. After the portio vaginalis had been brought into the speculum, the vagina was dried up and the measuring-cylinders passed through one of the cervical canals. If a certain cylinder did not pass smoothly through the canal, the next higher size was cautiously tried. If this could not be passed through without undue pressure, the thickness of the preceding cylinder that could was taken as the value of the canal calibre.

Experiments 1—8. Eight animals with a normal sexual cycle were followed from the pro-oestrous phase (first day, 1 p. m.) to the met-oestrous phase (second day, 7 p. m.). Guided by changes in the vaginal smear the calibre of the cervical canal was measured at different periods (Table 1).

Table 1.

Stage ¹	I										II										III				IV		
Phase ¹	Pro				P o s																Met						
Time of day	1	5	7	8	9	11	12	1	3	7	10	1	4	7													
	p. m.										a. m.				p. m.												
Exp.	Rat	Calibre of cervical canal in mm at above periods																									
1	824	0.5			0.8	1.0			1.5					1.5													
2	841	0.8			0.8	1.0			1.5					1.5													
3	849	0.8	1.0			1.0			1.5					2													
4	853	0.8		1.0					1.0			1.5		1.5													
5	860	1.0			0.8		1.0			1.5				1.5													
6	868	0.8			1.0			1.0		1.5				2													
7	873	0.8				1.0			1.0				1.5	1.5													
8	874	0.5				1.0		1.0		1.5				1.5													

Table 1 shows that in the pro-oestrous phase the cervical canal is only in exceptional cases (Exp. 5) greater in calibre than 0.8 mm, at times less. The canal gradually increases in calibre,

¹ 'Stage' and 'Phase' — for explanation see *Genell: Acta physiol. scand.* 1940: 1: 139.

so that during the first half of the oestrous phase (pos = Stage II) it measures about 1 mm. In the later half of the oestrous phase (= Stage III) it increases further to 1.5 mm. In the subsequent course of the sexual cycle (only partly recorded in the table) the cervical canal allows the passage of at least a 1.5 mm cylinder, sometimes a 2 mm one.

The cervical canal is invariably narrow during the whole of the pro-phase and beginning of the pos-phase, i. e. during a period of about 24 hours. It seems justifiable to interpret the variations in the calibre of the cervical canal as tonus variations in the unstriated muscle.

Experiments 9—14. Ten castrated animals. The calibre of the cervical canal was measured. Varying doses of oestrin were administered to the animals and the calibre of the cervical canal re-measured (Table 2).

Table 2.

Exp.	Rat	Cervical Canal:		Oestrin treatm.		Cervical Canal: calibre		
		Day after castr.	Calibre in mm	Day after castr.	Dose in I. U.	Day after castr.	Vaginal smear	in mm
9	944	7	1.5	7	15	9	neg-pro	1.5
10	946	7	1.5	7	15	9	neg-pro	1.5
11	952	7	1.5	7	30	9	(pro)	1.5
12	961	7	1.5	7	30	9	(pro)	1.5
13	963	7	1.5	7	45	9	neg	1.5
14	969	7	1.5	7	45	9	pro	1.5
9	944	14	1.5	14	150	16	(pos)	1.0
10	949	14	1.5	14	150	16	pro-po+	1.0
11	952	14	1.5	14	150	16	pro	0.8
12	961	14	1.5	14	300	16	pro	0.5
13	963	14	1.5	14	300	16	pro	0.8
14	969	14	1.5	14	300	16	pro	0.8

Table 2 shows that 7—14 days after the castration the calibre of the cervical canal is 1.5 mm. Oestrin doses of 15, 30 and 45

i. u. have no constrictive effect on the cervix, while doses of 150 i. u. reduce the calibre a little. Not until a dose of 300 i. u. (which has been experimentally shown to be the adequate oestrogenic dose for the rat, GENELL, 1937) has been reached, does the typical narrowing of oestrus to a calibre of 0.5—0.8 mm appear in the cervical canal.

From these experiments it can be seen that the tonus of the cervical muscles of the cervix is high during oestrus and low during dioestrus and in castrates. Treatment of castrates with an adequate oestrogenic dose of oestrin brings the cervical muscles into the tonic state characteristic of heat. The variations in the tonus of the cervical muscles during the sexual cycle are thus regulated by the oestrous hormone, oestrin.

Spontaneous Activity and Drug Reaction.

Fourteen animals have been tested. As the cervical musculature of the rat is almost exclusively circular, the cervix preparation was made in the following manner. The uterus and vagina were cut completely away. All the connective tissue of the parametrium was removed. The cervix pre-dissected in this way consists of a firm, conical body 7 to 8 mm long and 3—5 mm wide. It was sliced transversely into about 1-mm thick rings. At the two lowest of these the cervical canal is still single, at the upper it has forked. For recording purposes the lowest but one (N) and the uppermost but one (Ü) of these rings were selected. The customary dispositions for the Magnus-Kehrer experiment were adopted. Medium: 100 cc serum saline solution: NaCl 8, KCl 0.42, CaCl₂ 0.24, MgCl₂ 0.005, NaHCO₃ 1, glucose 0.5 gm per 1000 cc. Oxygen with 5 per cent CO₂ was bubbled through. Of the 14 experiments six are selected for reproduction /Figs. 1—6/.

Spontaneous Activity. In surviving preparations the circular muscle of the cervix, like the muscle of the uterine cornu, develops a spontaneous rhythmic activity. This shows a slightly greater contraction frequency and a slightly greater amplitude in the upper part of the cervix than in the lower. The differences between the sexual phases are less striking in the circular muscle of the cervix than in that of the cornu uteri.

In *pro-oestrus* (pro, Figs. 1, 2, 3) the rhythm seems to be somewhat more rapid than in *oestrus* (pos, Fig. 4, lower curve), *met-oestrus* (met, Fig. 5) and *dioestrus* (neg 4, Fig. 4, upper curve). In

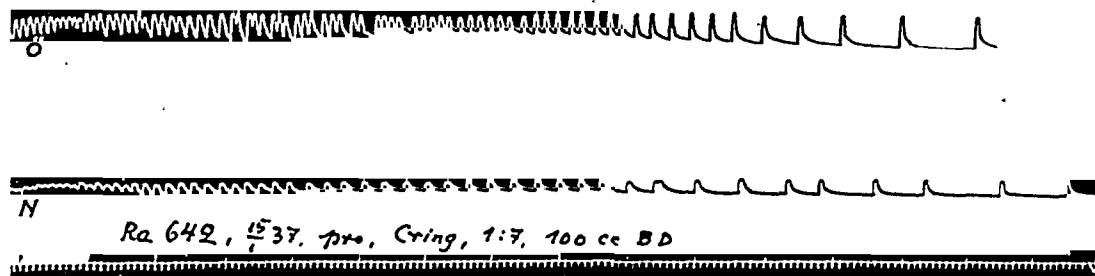


Fig. 1. Rat, pro-oestrus, cervix, circular musculature, upper (Ö) and lower (N) segment. Pen-stroke ratio 1:7. Medium 100 cc. Time in min.

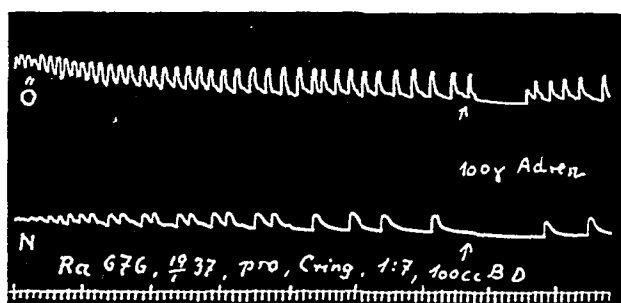


Fig. 2. Rat, pro-oestrus, cervix, circular musculature, upper (Ö) and lower (N) segment. Pen-stroke ratio 1:7. Medium 100 cc. Addition 100 γ adrenaline. Time in min.

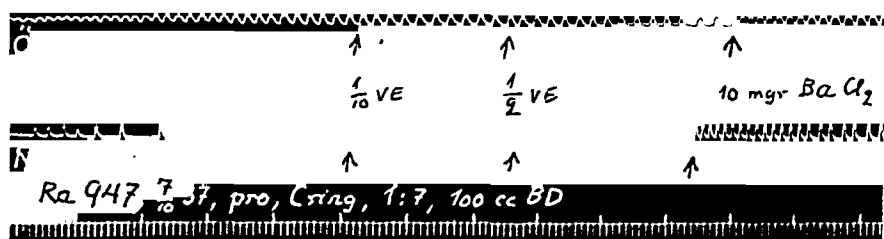


Fig. 3. Rat, pro-oestrus, cervix, circular musculature, upper (Ö) and lower (N) segment. Pen-stroke ratio 1:7. Medium 100 cc. Addition (from left) 0.1, 0.5 unit pitocin, 10 Mgm BaCl_2 . Time in min.

the three last-mentioned phases, moreover, the spontaneous activity thins out (and ceases) far quicker than in *pro-oestrus*. The difference between oestrus and dioestrus in the cervical muscles is accordingly the reverse of that observed in the rest of the uterine muscles of the rat (GENELL, 1936, 1937).

Castration brings about a profound change in the spontaneous activity. In the upper cervix this activity almost ceases, in the lower it ceases altogether (Fig. 6).

Drug Reactions. The cervical muscles show no response to *pitocin* (Figs. 3, 4), either by increase of contraction frequency and amplitude or by increase of tonus. In pro-oestrus (Fig. 3) an undoubted increase in the frequency of contraction is observed after a (unphysiologically) high dose of *pitocin* ($1\frac{1}{2}$ unit per 100 cc). Adrenaline has the same effect on the cervical muscles as on the rest of the uterine muscles: inhibition and depression of tonus (Fig. 2). BaCl_2 has the same effect on cervical muscle as it has on all other unstriated muscle (Figs. 3, 4, 5, 6).

Discussion.

Whereas the tonus of the rat's uterine muscle (GENELL, 1940) is high in the dioestrous phase and low in the oestrous, the reverse applies to the cervical muscles, as can be seen from the present investigation. Substitute experiments on castrates show that the change of tonus is evoked in both cases by oestrin. Thus, as regards muscle-tonus, the oestrous hormone of the ovary produces contrary effects in the cervix and corpus uteri. The spontaneous activity of the rat's uterus (corpus) is characterized by low frequency and great amplitude in the oestrous phase, by high frequency and small amplitude in the dioestrous phase (GENELL, 1936, 1937). The reverse state prevails in the cervical muscles. NEWTON (1933), working on surviving preparations from guinea-pigs and rats, showed that cervical muscle is refractory to the posterior-lobe hormone. This fact is also brought out by the present investigation. A condition contrary to that in the muscular apparatus of the corpus uteri is also present here. On the other hand, the cervix does not respond to adrenaline in the same manner as the rest of the uterine muscles.

Summary.

In vivo-investigations of the tonus conditions of the cervical muscles have shown that the tonus is highest in the oestrous phase, lowest in the dioestrous phase and after castration. In substitute experiments it has been shown that the increase of tonus in oestrus is elicited by oestrin. Surviving prepara-

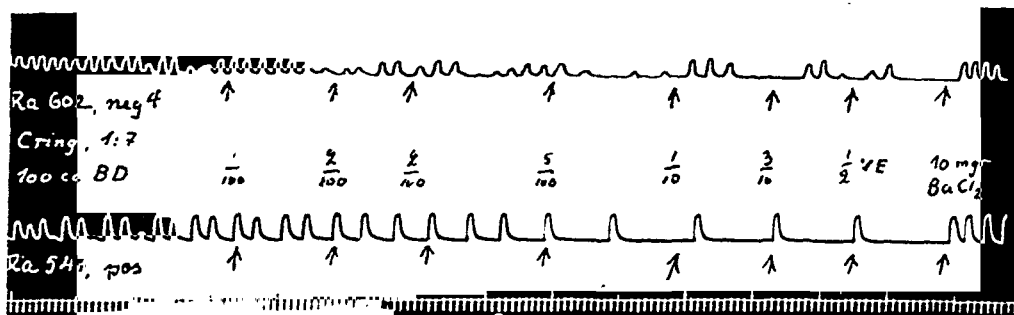


Fig. 4. Upper curve: rat, dioestrus, cervix, circular musculature. Lower curve: rat, oestrus, cervix, circular musculature. Pen-stroke ratio 1:7. Medium 100 cc. Additions (from left) 0.01, 0.02, 0.02, 0.05, 0.1, 0.3, 0.5 unit pitocin, 10 Mgm BaCl_2 . Time in min.

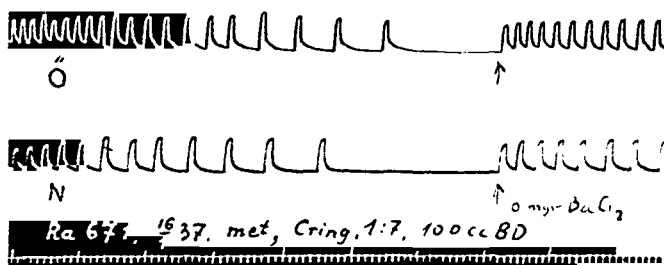


Fig. 5. Rat, metoestrus, cervix, circular musculature, upper (Ö) and lower (N) segment. Pen-stroke ratio 1:7. Medium 100 cc. Addition 10 Mgm BaCl_2 . Time in min.

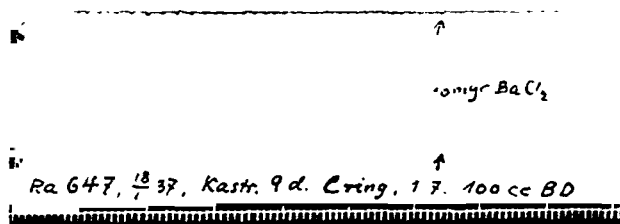


Fig. 6. Rat, castrated 9 days earlier, cervix, circular musculature, upper (Ö) and lower (N) segment. Pen-stroke ratio 1:7. Medium 100 cc. Addition 10 Mgm BaCl_2 . Time in min.

tions of circular muscle from the cervix have been studied in different sexual phases. In the oestrous phase the spontaneous activity is of quicker rhythm and greater duration than in the dioestrous. After castration the spontaneous activity tends to cease.

Pitocin is without effect on the cervical muscles.

Adrenaline causes inhibition and depression of tonus in the cervical muscles.

The cervical musculature, in respect of spontaneous activity and tonus, behaves inversely to the rest of the uterine musculature, and in contrast to this is refractory to pitocin but like this is negatively adrenotropic.

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Rotation of Activity and Spontaneous Rhythms in the Retina.¹

By

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(With 4 figures in the text.)

Several years ago (GRANIT and THERMAN, 1935) we made the first observations on rotation of activity in the eye. Later this same phenomenon turned up in some experiments with constant stimuli as an alternation between types of electroretinograms so different, that we felt compelled to speak of a "switchboard" in the retina (GRANIT and MUNSTERHJELM, 1937). With rhythmic stimuli rotation of activity has also been observed by BARTLEY (1937) and recently BARTLEY (1939) and BERGER and BUCHTHAL (1938) have emphasized the general significance of this phenomenon in the physiology of vision.

When I now return to this subject it is partly because our present micro-electrode technique (GRANIT and SVAETICHIN, 1939) brings it out with hitherto non-paralleled clarity and regularity, partly because of my conviction that rotation of activity is a very essential property of the nervous system.

From the point of view of the general physiology of the special senses one would be prepared to state that there are good reasons for accepting as a law of end-organ differentiation that the evolution of structurally more complicated sense organs takes place in such a manner as to counteract the effects of adaptation with their tendency to make the stimulus ineffective. Rotation of activity is one of the means whereby this end is attained. The retina which is forced to continuous, accurate activity within an

¹ Received 2 January, 1941.

enormous range of illumination has this mechanism well developed and, in addition, possesses several other means of suppressing or modifying the effects of adaptation. These, however, cannot be discussed now.

Technique and Procedure.

The experiments were carried out with the silver-micro-electrode described by GRANIT and SVAETICHIN (1939). In many cases the glass-covered platinum micro-electrodes of TAYLOR and WHITAKER (1928) were used, yet without making them non-polarizable. Experiments proved, as was to be expected, that the metal-micro-electrode has a polarization capacity which greatly deforms the slow retinal action potential without preventing the fast spikes from appearing. If the glass covered metal-micro-electrode be used with a directly coupled amplifier it is found to record a rectangular input current with the distortion characterizing a condenser-coupled instrument having somewhat larger coupling condensers than those used in my amplifier for micro-recording of spikes from the retina.

The micro-electrode is applied on to the retina with the aid of a micro-manipulator under a binocular microscope. Leads are taken to a condenser-coupled amplifier with a balanced input stage. A cathode ray and a loudspeaker are connected to the output stages in the usual manner. When the light is switched on or off discharges composed of spikes follow from elements which are more or less isolated as the case may be (see figures). These remind one of the spikes recorded with micro-electrodes, for instance, by FORBES and his collaborators RENSHAW, THERMAN ET AL. (1937, 1940, 1940) from the hippocampus area and by LORENTE DE NÓ (1939) from the nuclei of ocular nerves.

GRANIT and SVAETICHIN (1939) held these spikes to arise from the neurite not too far from the axone hillock of the retinal ganglions. The reason for this, not mentioned in their paper, was that spikes could be obtained when their micro-illuminator was pushed into the retina several millimeters away from the micro-electrode. According to HARTLINE (1939) the "receptive field" of a single fibre would have much narrower dimensions and the retina itself is only a fraction of a millimeter. On the other hand the spikes cannot arise too far away from the ganglions as they are absent or minute in the blind spot.

In the experiments to be described below the *whole retina* was illuminated with some wave-length from our monochromator. For technical details the reader is referred to the paper by GRANIT and SVAETICHIN (1939). The experimental animals were frogs and tortoises.

Results.

The Response to Intermittent Stimuli.

Rotation of activity is conspicuous and most disturbing in experiments presupposing a constant threshold. To this type belongs the colour work taken up by GRANIT and SVAETICHIN [1939] and at present continued in this laboratory with a variety of animals. Sometimes one finds the threshold to undergo sudden changes which could be ascribed to the pressure of the micro-electrode were it not for the fact that these changes may come and go without, in the long run, involving any progressive diminution of the sensitivity. In fact, I have followed dark-adaptation for two hours with a well placed micro-electrode. That pressure *can* lead to spontaneous discharges is a different matter. But this source of error can with some experience be avoided and then the sudden shifts in the level of sensitivity must be ascribed to causes in line with those leading to the spontaneous rhythms to be described in the next section. These factors cannot, as a rule, be put under experimental control, though the phenomena are interesting to record and try to modify when they occur.

But inasmuch as rotation of activity depends on stimulation, which may be supposed to activate after-potentials or other mechanisms of blocking or facilitation, then one certain way of regularly bringing these into play would be intermittent illumination. Every flash of light then leads to a state of excitation which has to force its way through a bed of receptors and neurons modified in excitability by the foregoing flashes. Records, from such experiments are shown in Fig. 1.

From above downwards I have selected cases designed to illustrate the rotation of activity of a gradually increasing number of neurons. There are also variations in the frequency of the flashes and in their strength. The essential features of the phenomenon are displayed, independently of the conditions chosen. The units come and go in a rotation of activity which is irregular with respect to the rhythm of stimulation. The active unit of

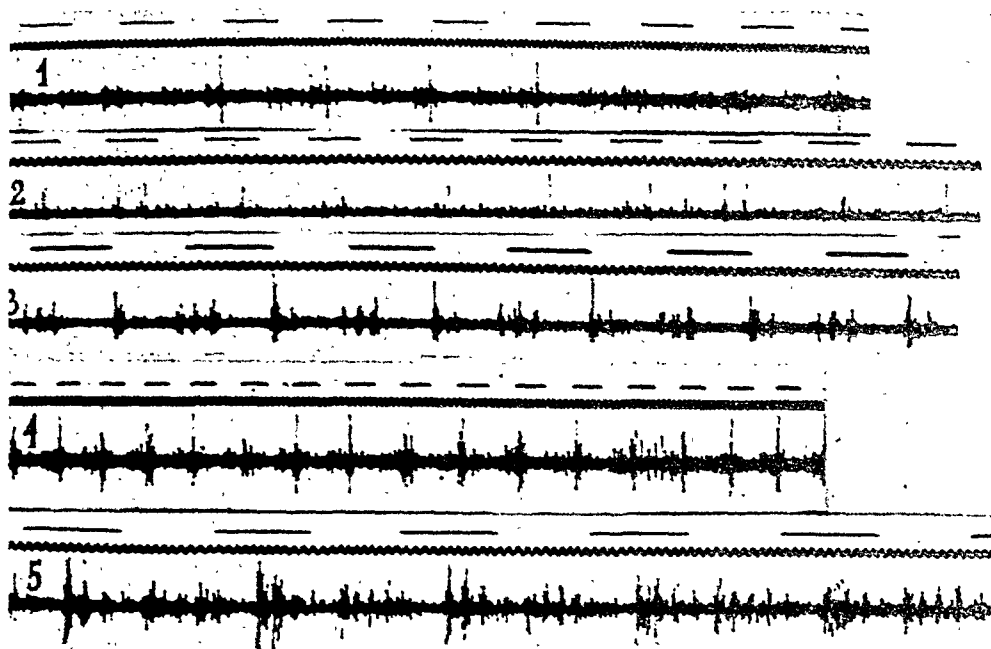


Fig. 1. The response to intermittent light at different frequencies. Light signal above time signal (50 per sec.) in this and the following records.

1. Tortoise, wave-length 0.680 μ . "Off"-spikes.
2. Frog, wave-length not noted.
3. Frog, spikes at both "on" and "off". Near threshold for wave-length 0.530 μ .
4. Tortoise, wave-length 0.620 μ .
5. Frog, wave-length 0.600 μ . Well above threshold for intermittent light.

curve 1 pauses every now and then, and again returns after a few flashes. In curve 2 there are three units fairly well placed relative to the electrode and the picture in this case as well as in curves 3—5 with a greater number of elements in activity is already very complicated. Continued stimulation with intermittent light neither seems to make the rotation of activity more regular nor does it abolish it.

Spontaneous Rhythms.

ADRIAN and MATTHEWS (1928) made the first observations on spontaneous rhythms and synchronization in the retina when recording from the whole nerve. The off-effect is quite often synchronized (GRANIT and THERMAN, 1935), particularly in the fibres that merely react to cessation of illumination (HARTLINE, 1938).

With the micro-electrode technique spontaneous discharges are quite common and very often these consist of grouped spikes

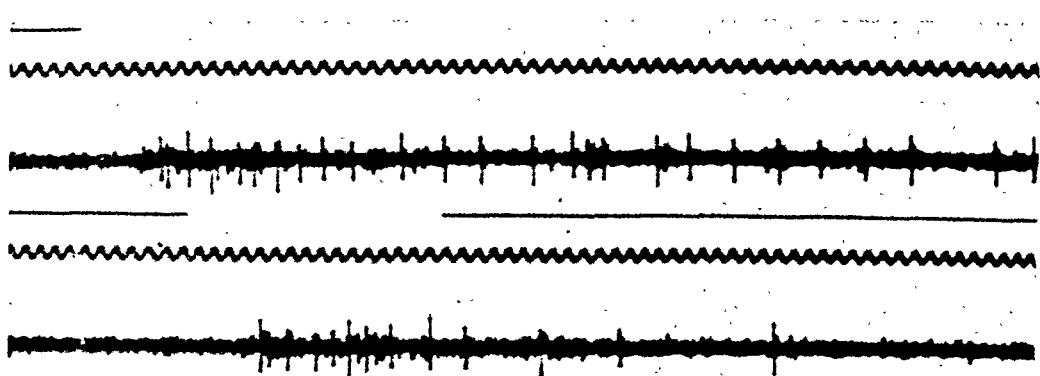


Fig. 2. Tortoise. Upper curve: off-effect control.
Lower curve: inhibition caused by re-illumination. Wave-length 0.600μ , near threshold.

which for some time may be synchronized. In connection with the problem of rotation of activity it interested me to find out to what an extent such discharges and rhythms, when they occur, can be modified by stimulation.

Very variable, though in each case repeatable, results were obtained. At one end of the series of observations could be placed the ordinary inhibitable off-effect, illustrated in Fig. 2. Somewhat similar properties has the spontaneous discharge of the dark-adapted eye, particularly when it is diffuse and not synchronized. With the frog's eye it is often a sign of dark-adaptation that the retina begins to discharge spontaneously. This discharge, as a rule, is very effectively inhibited by illumination. "Dark" rhythms of the eye of the water-beetle (*Dytiscus marginalis*) behave similarly, according to ADRIAN (1937).

An interesting case is curve 1 of Fig. 3. There was a spontaneous discharge having the fairly regular rhythm illustrated. A light flashed into this discharge (see the curve) led to a temporary inhibition probably caused by the on-discharge elicited in the neighbourhood. Signs of this on-effect are seen under the micro-electrode. Cessation of stimulation was followed by a brief off-discharge and another temporary silent period after which the receptor again picked up its original rhythm. The rhythmic discharge lasted for some time so that the experiment was several times repeated. The results show that cessation of illumination also can exert inhibitory activity. This hitherto seemed to be a prerogative of illumination falling into an off-discharge. The

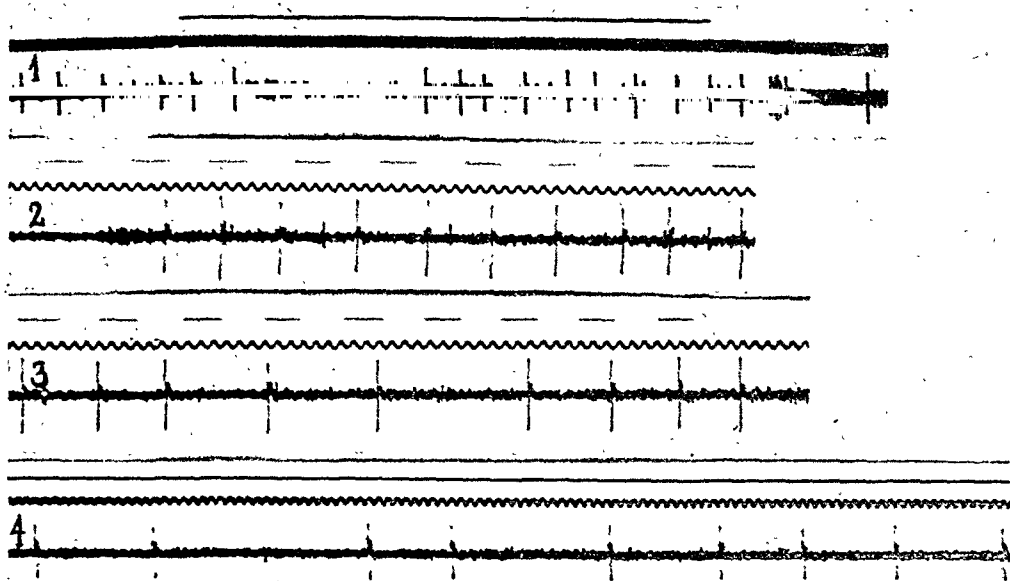


Fig. 3. 1. Tortoise. Spontaneous rhythm inhibited by onset and cessation of stimulation with wave-length 0.620μ at maximal intensity of monochromator. 2 and 3. Tortoise. Wave-length 0.620μ , well above threshold (see text). 4. Tortoise. Active unit firing spontaneously also during illumination.

Note. The single units illustrated in these experiments are the "red" receptors of the tortoise with maximal sensitivity around 0.620μ (GRANIT 1941).

inhibition at "off" is probably to be parallelized with the well-known "silent periods" of other receptors.

The counterpart to curve 1 of Fig. 3 is curve 4. This shows the spontaneous activity of a single element which seemed to be quite independent of whether the eye was illuminated or not. This is nearly always the case with a discharge caused by the pressure of the micro-electrode. But here there was no reason to suspect that mechanical stimulation had caused the activity. When slowly screwing the micro-electrode into position and looking at it all the time in the microscope one often first hears the rhythm in the loudspeaker as a faint distant noise which then gradually increases in strength as the point of the electrode approaches the active unit from above. When this is so there is no reason to ascribe the discharge to mechanical stimulation, the less so as the spontaneous activity quite often may cease as suddenly as it has begun. When light does not influence such rhythms the reason may have been that the monochromator gave too feeble stimuli. But there was always a check on this in the

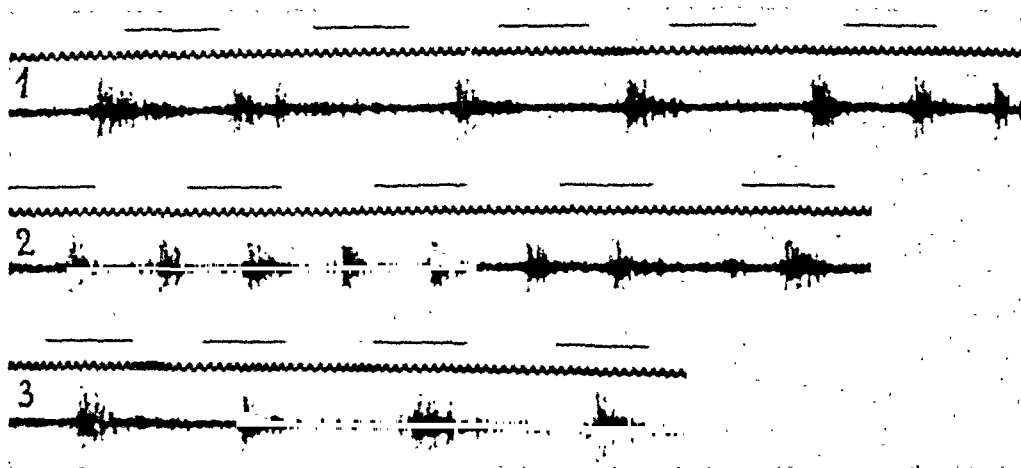


Fig. 4. Frog's eye which has been in the apparatus for 1 h. 40 min. During this time it has dark-adapted and begun to discharge spontaneously 15—18 times a minute. The active region is of the type giving a "grouped discharge", which comes above and goes below the threshold as a grouped unit when the intensity is varied. The spontaneous discharge looks like the discharge caused by stimulation. Wave-length 0.520μ . For explanation, see text.

ab. 2,400 m. c. light of the lamp of the preparation microscope illuminating the retina from above. This light could be switched on and off as a final control on the relative independence of the rhythm.

There are undoubtedly, particularly in the eye of the tortoise, spontaneous rhythms which behave as if they had succeeded in blocking every path around the discharging units.

Curves 2—3 of Fig. 3 show the beginning and end of intermittent stimulation with the micro-electrode in a place which at the moment of recording was silent, but some minutes later began to discharge spontaneously. At the moment of recording the tendency to spontaneous activity probably was *in statu nascendi* and its appearance was facilitated by the flashes from which the rhythm of the discharge rapidly disconnected itself.

In such cases the result may become very complex. Fig. 4 illustrates an eye tending to give a grouped, spontaneous discharge very much like the ensuing response to flicker. The active spot is silent when the recording begins and, upon illumination, follows the slow rhythm of the intermittent stimulus. Spontaneous activity begins soon (end of curve 1) but immediately is pressed into the rhythm of the stimulus where (curve 2) the extra

discharge takes up a definite place. In curve 3 it has disappeared and the eye now again reacts as in the beginning (curve 1).

These experiments, chosen to illustrate some properties of spontaneous rhythms in the retina, could easily be multiplied and would then provide samples of most of the phenomena of a similar nature observed in the central nervous system. But as they seem to have relatively little analytical value in their present form I have merely used them as a complement to the observations on the rotation of activity. From this point of view their significance is that they illustrate how inhibition can block a discharge of spikes and how rhythms imposed by a stimulus can play upon rhythmic tendencies in a given group of neurones.

Discussion.

It is clear that excitation, spontaneous or caused by stimulation, is surrounded by inhibitory and excitatory influences spreading over the retina in complicated patterns. These may well be of the nature of after-potentials. The net result of the waxing and waning of such effects must lead to rotation of activity among the active elements. There is also at the threshold a fluctuation of excitability which may or may not be of different origin, but whether it is of any significance with "flicker" is open to doubt. In order to make a group of spikes follow an intermittent stimulus the illumination must be well above the threshold for a constant light.

For the physiology of vision the rotation of activity, apart from what it may do to counteract the affects of adaptation, is of great interest and emphasizes points of view advocated by BARTLEY (1939), BERGER and BUCHTHAL (1938) and WRIGHT and GRANIT (1938).

Summary.

"Spikes", recorded from the retina with micro-electrodes and a condenser-coupled amplifier, are elicited by stimulation with intermittent light.

Single units of activity as well as a greater number of active elements responding to intermittent light, show a very marked

rotation of activity, the individual elements pausing and re-entering into activity at irregular intervals.

Spontaneous activity in the retina can with respect to an interposed stimulus be divided into two categories, (i) discharges which can be temporarily or completely inhibited by illumination or even temporarily inhibited by cessation of illumination, and (ii) discharges which continue independently of whether the eye is illuminated or not.

Spontaneous rhythms can be activated and facilitated by rhythmic stimulation.

The experiments thus give further support to the view that waves of excitation passing through the retina are surrounded by spreading patterns of inhibitory and excitatory influences. With stimuli well above the threshold, these probably are the main causes of the rotation of activity.

From the sensory point of view rotation of activity may be regarded as one of the means whereby the effects of adaptation are counteracted, and it also emphasizes the fundamental truth behind "pattern" theories of visual processes.

The experiments have been carried out with the support of a grant from The Rockefeller Foundation to this laboratory.

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1938.
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From The Neurophysiological Laboratory, The Caroline Institute,
Stockholm, and The Anatomical Institute of
Helsingfors University.

Dark-Adaptation and the Platinum Chloride Method of Staining Visual Purple.¹

By

STEN STENIUS.

(With 1 Fig. in the text.)

From different points of view GRANIT ET AL. (1938, 1939) and LYTHGOE ET AL. (1936, 1940) have arrived at the conclusion that visual purple acts along the surface of the rods where, accordingly, its concentration should be maximal.

This conclusion formed the starting point of my attempt to try STERN's (1905) platinum chloride method for the staining of visual purple in order to find out how the substance is distributed. STERN's original description does not give the details of his procedure, but in a personal communication to Prof. GRANIT some valuable suggestions were made by Dr. TANSLEY who also has used this method (1933). Some 40—50 eyes were spent in my own work and the procedure that finally emerged from these trials led to good and repeatable results. Description of this method should save a great deal of labour for anyone wanting to apply it.

The frogs (*Rana esculenta*) were dark adapted and decapitated in red light, the eyes excised and freed from all slices of adjoining tissue. For each eye 2 cc of a 2½ % platinum chloride solution were used. The best method proved to be injection of the fixative through the optic nerve into the bulb with the aid of a fine needle.

After this the eyes were left for not less than 12 hours in the platinum chloride solution. In this manner bleaching of the visual purple was prevented before fixation had taken place.

¹ Received 17. November 1940.

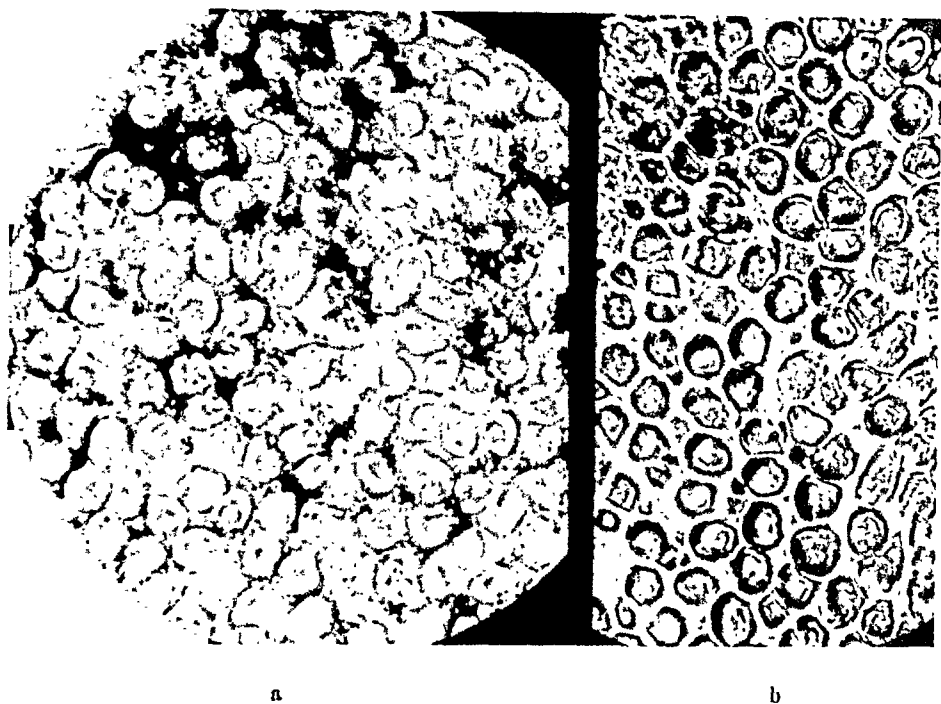


Fig. 1. Microphotographs of cross sections of frog retinæ in the dark-adapted state fixated (a) in platinum chloride, (b) in Zenker's solution. Zeiss ultraphot.

Then the eyes were cut open and the anterior half of the bulb removed. Then followed fixation in absolute alcohol for 1 hour, the fluid being renewed after half an hour. The next step was clearing in xylol for half an hour, the fluid being renewed after 15 min. Finally the eyes were soaked for one hour in melted paraffin (52°) and embedded in the paraffin of a second bath (58°) where they also had been for one hour.

Sections at 10 μ did not change their colour during the first few days if they were kept in the dark. In daylight the yellow colour gradually disappeared (but see STERN, 1905).

Of greatest interest proved to be the cross sections of the rods illustrated in fig. 1a. Fig. 1b is a control fixated in ZENKER's solution. The rods fixated in platinum chloride were surrounded by a yellow highly refractive ring or horse-shoe of visual purple, whereas the outer limbs of rods fixated in ZENKER's solution (fig. 2) looked like compact discs inside a girdle of more refractive material. The horse-shoe or ring of visual purple, stained yellow, does not show unless the visual purple has a fairly high

concentration. Eyes in different stages of regeneration were taken but it was not possible to demonstrate an intermediate stage between the "horse-shoe" and no regeneration.

When the rods were cut along their long axis in the usual manner their outer limbs stained with platinum chloride looked "inflated" compared with those fixated in ZENKER's solution. The yellow colour appeared unevenly distributed giving the impression of the rods being hollow or perforated.

The cross sections certainly did give a picture of distribution of visual purple which is in complete accordance with the views held by GRANIT and LYTHGOE. On the other hand, it should be remembered that platinum chloride penetrates relatively slowly. This may lead to the surface being stained before the interior of the cell which again may prevent further penetration of the fixative into the latter. But if this were the case, it would be difficult to explain why the majority of the rods are surrounded by "horse-shoes" of stained visual purple in which the open end of the horse-shoe often is turned towards the stained portion of an adjacent cell. One would then be forced to assume that the "horse-shoes" depended on ruptures of originally complete rings during some phase of the process.

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Über den Reizmechanismus der Chemorezeptoren im Glomus caroticum.¹

Von

U. S. v. EULER, G. LILJESTRAND and Y. ZOTTERMAN.

(Mit 1 Fig. im Texte.)

HEYMANS, BOUCKAERT und DAUTREBANDE stellten 1931 fest, dass gewisse atmungserregende Gifte wie Lobelin und Nikotin ihre Wirkung reflektorisch über das Carotissinusgebiet ausübten. Ähnliches wurde später auch für Azetylcholin (HEYMANS u. Mitarb. 1936) und Kaliumionen (EULER, 1938) gefunden. Die genannten Wirkstoffe sind alle durch ihre synaptotrope Wirkung gekennzeichnet, und es wurde die Annahme vorgeführt, dass sie prinzipiell gleichartig, d. h. erregend an synapsähnlichen Strukturen, wirken (EULER, LILJESTRAND und ZOTTERMAN, 1939).

Wenn Aktionspotentiale vom Carotissinusnerven der Katze abgeleitet werden, beobachtet man nach Zufuhr von Lobelin oder Nikotin eine starke Zunahme der Impulsfrequenz, die sich auf die feineren, chemische Impulse tragenden Fasern bezieht. Die grösseren Druckimpulse bleiben dabei unbeeinflusst. In neueren Versuchen haben wir nachgewiesen, dass auch kleine Mengen Azetylcholin, die am atropinisierten Tier keine Atmungs- oder Kreislaufwirkung hatten, eine bald vorübergehende aber starke Erhöhung der Impulsfrequenz herbeiführten. In diesen Versuchen wurde 5—10 μg durch die A. carotis externa dem Sinusgebiet zugeführt. (Fig. 1).

Dieses Ergebnis scheint die Möglichkeit auszuschliessen, dass es sich um eine direkte Erregung der Chemorezeptoren handeln

¹ Eingegangen am 14. Dezember 1940.

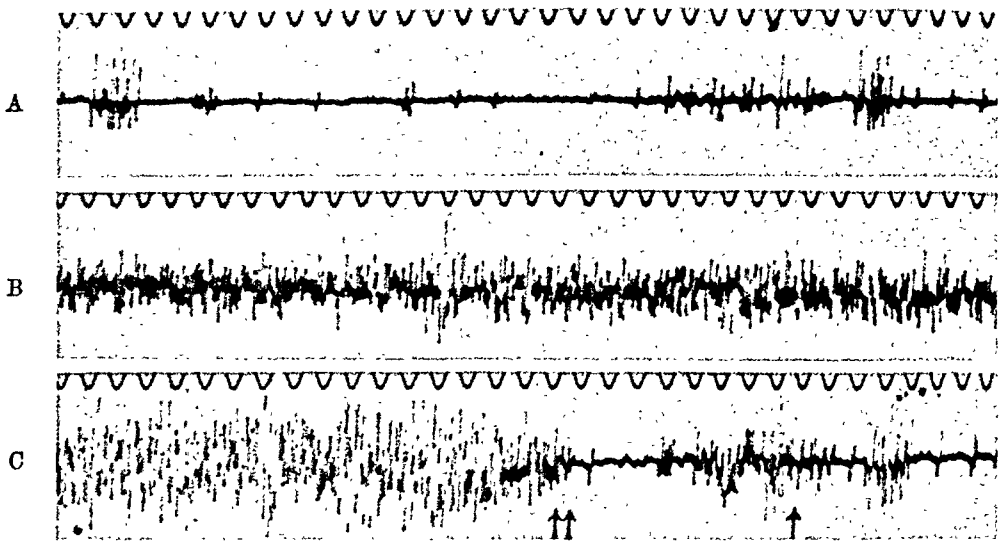


Fig. 1. Carotissinusnerv, Katze, Chloralose. 1 mg Atropinsulfat pro kg intravenös. A. Chemorezeptoren durch Überventilation ausgeschaltet. B. Reichlich chemische Impulse nach Injektion von 7 μ g Azetylcholin in 0.15 ml Ringerlösung durch die A. carotis externa. C. Einsetzen der Azetylcholinwirkung bei $\uparrow\uparrow$, 0.16 Sek. nach der Injektion bei \uparrow . Kurven von rechts nach links zu lesen. Zeit $\frac{1}{50}$ Sek.

könne, da Reizwirkungen von Azetylcholin an sensiblen Nervenendigungen unwahrscheinlich und zumindestens nicht bekannt sind, im Gegensatz zu Lobelin, Nikotin und Kaliumionen. Andererseits besitzt Azetylcholin eine charakteristische Reizwirkung an Synapsen verschiedener Art, wie sie durch die Ergebnisse über die Erregungsüberleitung in sympathischen Ganglien und motorischen Endplatten bekannt ist.

Wir sehen hierin eine Stütze für unsere Auffassung, dass nicht nur Azetylcholin sondern auch die übrigen, oben erwähnten chemisch aktiven Stoffe durch eine synaptotrope Wirkung ihren Effekt entfalten. Das Vorhandensein von Ganglienzellen im Glomusgebiet steht hiermit in Einklang.

Eine Erregung der Barorezeptoren konnte mit Azetylcholin nicht beobachtet werden.

Durch die hier mitgeteilten Befunde dürfte somit erstmalig ein Synapsmechanismus in afferenten Nerven ausserhalb der Sinnesorgane dargetan sein.

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The "Red" Receptor of *Testudo*.¹

By

RAGNAR GRANIT.

(With 1 figure in the text.)

By means of the micro-electrode technique, applied as described in detail by GRANIT and SVAETICHIN (1939), I have recorded with cathode ray and condenser coupled amplifier the "spikes" of activity from single units in the cone-retina of the tortoise (*Testudo graeca*). It was found to be particularly easy to isolate a "red" receptor in this eye, in fact, the records of single units in Fig. 3 of my recent paper in the same volume of This Journal (GRANIT, 1941) are all samples of this "red" receptor. The whole retina has been illuminated with light from a Tutton monochromator, and the energy measured necessary for a constant response such as the absolute threshold or the cessation of "flicker", caused by intermittent stimuli. For energy control etc., see the paper by GRANIT and SVAETICHIN (1939).

The curve drawn in full between the large circles in Fig. 1 shows inverse relative energies in per cent of the maximum, placed in 0.620μ . Behind this curve are the averages of 81 readings with 5 animals. With three of them the absolute threshold for a single "on"-spike was studied, with the other two the cessation of "flicker" for respectively an "on"- and an "off"-spike was the constant index necessary for the measurements. The curve was independent of the index used. The eyes were in different states of adaptation but this factor also had no influence on the distribution of sensitivity of the "red" receptor. The state of adaptation merely determines the general level of excitability.

¹ Received 2 January, 1941.

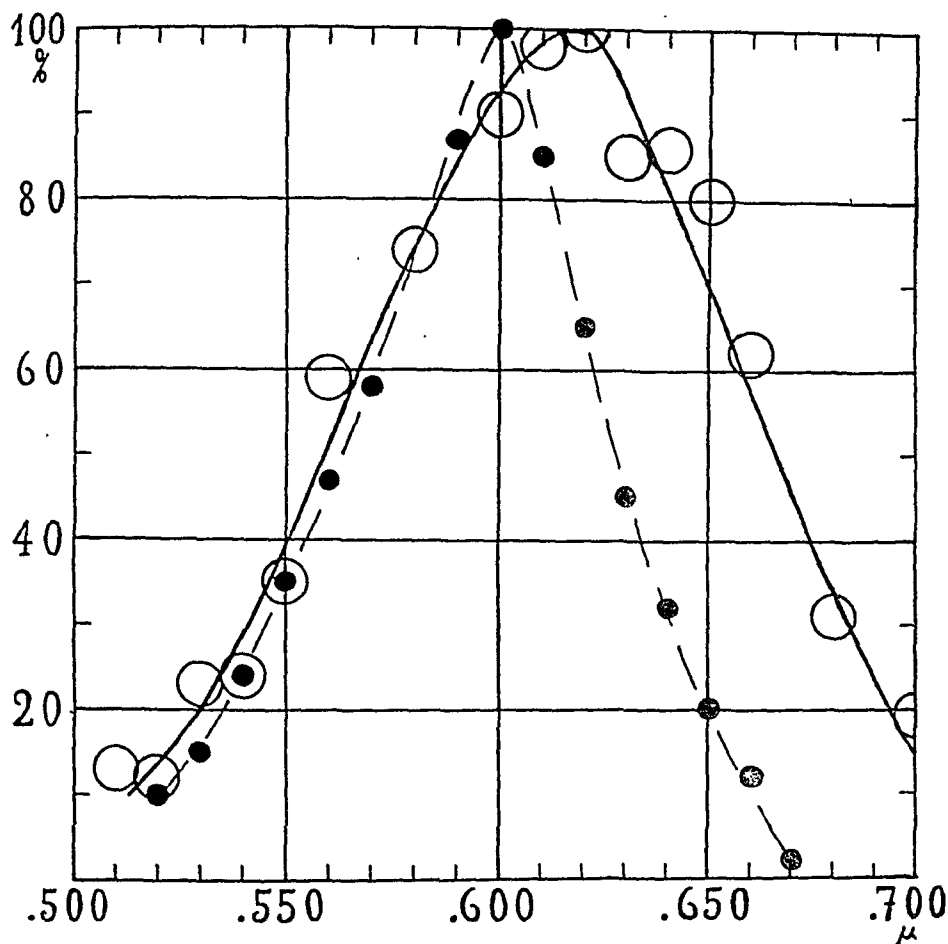


Fig. 1.

In this respect the curve for the "red" receptor radically differs from most of the visibility curves, obtained from lightadapted eyes of frogs. Nearly all receptors which in the photopic frog's eye are sensitive to long wave-lengths become greensensitive during dark-adaptation and finally their visibility curves acquire the shape of absorption curves for visual purple (GRANIT and SVAETICHIN, 1939) with maxima around 0.500μ .

For comparison I have added in dotted lines between the small filled circles the "reddest" receptor that I have found in the light-adapted frog's eye. Two spots in the same eye gave this narrow, unusually stable and precise visibility curve, based on a group of 3—4 very large spikes. With the electrode in the first spot were obtained 20 readings during 50 min., from the second

spot 29 readings during 32 min. The maximum of the 49 averaged observations is in 0.600μ , a somewhat unusual result, as the sensitivity maxima of receptors in the photopic frog's eye rarely go beyond 0.580μ and, as a rule, are gathered around 0.560μ .

The remarkable red-sensitivity of the most common type of receptor in the cone-retina of the tortoise also dominates visibility curves based on diffuse discharges made up of several active units. Under such conditions the maximum in the eyes of light-adapted frogs is between 0.550 — 0.560μ .

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An Attempt at Isolating the Carbohydrate Moiety of Crystalline Ovalbumin by Means of Cataphoresis.¹

By

ERIK JORPES and TORSTEN THANING.

The question regarding the possible carbohydrate content of the protein molecule has for many proteins been solved through their crystallization. On recrystallization the sugar moiety disappears. This has been the case with hemoglobin, edestin, pepsin, trypsin, carboxypolypeptidase, Bence Jones protein, insulin, secretin, urease and papain. Because of the specific nature of the proteins it seems reasonable to assume that the carbohydrate component occurring in many of them is an impurity.

None the less, the polysaccharide of ovalbumin cannot be removed by means of crystallization. The carbohydrate content remains constant, about 1.8 per cent, in spite of 8 to 10 recrystallizations (NEUBERGER, 1938).

We have tried, without success, another way of separating the two components. We have submitted the crystalline ovalbumin to cataphoresis in different buffer solutions. Contrary to expectation, the carbohydrate migrated with the protein both at pH 3.56 and at 13.2. Of particular interest is the fact that the two components followed each other closely even at pH 10.1 and 13.2 outside the stability region of the ovalbumin molecule (SVEDBERG, 1939).

The crystalline ovalbumin was recrystallized three times. The cataphoresis was performed in the usual way in the TISELIUS apparatus at 0°. Since the mobility of the ovalbumin molecule

¹ Received 16 January, 1941.

in the electric field at different pH values is well-known from earlier work, no attention was paid to this question. The sharp line was followed for 2 to 6 hours until the migrating solution almost filled the upper cell. On the contents of the different cells mikro-Kjeldahl and the quantitative Tillmans-Philippi orcin reaction were made. No stress was laid upon a quantitative determination of the sugar in the ovalbumin. The relative sugar content of the different cells was determined and compared with their nitrogen content.

In all experiments, amounting to 8 in all, with buffer solutions of pH between 3.56 and 13.6, the sugar followed the nitrogen as closely as one could expect, if allowance is made for the error inherent in the method for the determination of the sugar.

A similar finding was made (EDMAN and JORPES 1941) on submitting a highly purified, non-crystalline β -glukosidase solution of emulsin to cataphoresis. Here also the sugar, amounting to 5 to 6 per cent of the protein, closely followed the nitrogen at such different pH values as 3.78 and 8.72.

The investigation was aided by a grant from the Therese and Johan Andersson Memorial Foundation.

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ACTA PHYSIOLOGICA SCANDINAVICA

VOL. I. SUPPLEMENTUM I.

From the Physiological Department (Head: U. v. EULER) and the
Pediatric Clinic (Head: A. LICHTENSTEIN) of the Caroline Institute,
Stockholm, and the Physiology Institute (Head: R. GRANIT),
Helsingfors University.

CONTRIBUTIONS TO THE NEUROPHYSIOLOGY OF THE OPTIC PATHWAY

ACADEMICAL TREATISE

WHICH BY DUE PERMISSION WILL BE PUBLICLY DEFENDED AT THE CAROLINE
INSTITUTE, STOCKHOLM, ON MAY 14, 1940, AT 10 A. M.

IN THE LECTURE THEATRE OF THE PATHO-
LOGICAL DEPARTMENT

BY

CARL GUSTAF BERNHARD

MED. LIC.

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CARL GUSTAF BERNHARD.

PART I.

On Slow Potential Changes Following Light Stimuli in the Retina and the Optic Nerve of Frog.

Introduction.

From a neurophysiological point of view the retina is an especially interesting object, since it combines an extremely differentiated sense organ with a "true nervous centre" (CAJAL, 1894). The electrophysiological analysis of the retina and the optic nerve which has been made during recent decades (especially by ADRIAN and GRANIT *et al.*), has brought to light facts of considerable importance for the analysis of problems concerning the physiology of vision, as well as for the elucidation of certain central nervous mechanisms.

The phenomena of the SHERRINGTON school termed spatial and temporal summation, synchronization and inhibition, have their electrophysiological correlates in the retina and have been analysed. The distribution of the integrative processes in the retina in relation to its morphological features gives an example of how a functional analogy to the anatomical facts, as expressed in CAJAL's definition, has been proved. The results, which show that the retinal transition from scotopic to photopic vision is largely caused by changes in the neurological state of the retina, as well as by changes in the photochemical processes, may serve as an example of newly discovered facts of fundamental importance in the physiology of vision.

The investigations made by ADRIAN and MATTHEWS (1927 a and b, 1928) of the action potentials in the retina and the optic nerve of the eel were the first to give electrophysiological proof of the *synaptic functions of the retina*. They found in the optic nerve the general relation between the intensity of stimulation,

and the frequency of discharge which had been found to exist in other sense organs and neurones (see ADRIAN, 1932 a). In the increase of frequency and the shortening of latency they also found that an increase of the illuminated area was equivalent to an increase of the intensity of illumination. They made use of the influence of this spatial effect on the latency as a measurement of the interaction in the retina, and thus proved (1928) that the latency of the impulses in the optic nerve was shorter if 4 spatially separated areas were illuminated simultaneously than if each spot were stimulated separately. GRAHAM's experiments (1932) on the *Limulus* eye, which is lacking synapses, point to the fact that this spatial effect is a result of interaction transmitted by the lateral connections of the retina (ADRIAN and MATTHEWS 1927 a and b, 1928; GRANIT 1933). In this eye a shortening of the latency of the optic impulses is obtained by the increase of intensity, while a change of the area illuminated has no effect. ADRIAN and MATTHEWS (1928) showed on the spatial effect that the lateral connections in the eye react to strychnine in the same manner as similar connections do in the central nervous system.

Such experimental criteria for the synaptic activity of the retina augment the analytical importance of the complex slow retinal potential following illumination which is associated with impulse outburst in the optic nerve.

Slow potentials have now been found in many ganglion cell structures elsewhere. Associated with impulse discharge in efferent neurones, these potentials reflect in their courses different phases in the course of central excitation.

Thus, slow ganglion potentials have been found in the optic and abdominal ganglion of the water-beetle (ADRIAN, 1931, 1932 b and 1937 a), the spinal cord (GASSER and GRAHAM 1933; HUGHES and GASSER, 1934 a and b; ECCLES and PRITCHARD, 1937; HUGHES *et al.*, 1937; MATTHEWS, 1937; BARRON and MATTHEWS, 1936 and 1938), the superior cervical ganglion (ECCLES, 1935 a and b), the ciliar ganglion (WHITTERIDGE, 1937), ganglions of median cardiac nerve of *Limulus* (HEINBECKER, 1936), the inferior mesenteric ganglion (LLOYD, 1937 and 1939) and the fifth lumbar ganglion (OBRADOR and ODORITZ, 1936).

The slow potential appearing in the retina following illumination, *i. e.* the *electroretinogram*, (ERG) was first observed by FRITHIOF HOLMGREN 1865 (1880), and later, independently, by

DEWARE and MCKENDRICK (1873). GOTCH's records (1903) of the ERG were the first to give a true picture of all the different phases of the retinogram in agreement with later and more complete investigations. The proof that the principal course of the ERG is the same in all vertebrate eyes (BRÜCKE and GARTEN, 1907; PIPER, 1911; HARTLINE, 1925) is of co-ordinate importance, the corneal electrode in the principal phase of the retinogram being positive in relation to the electrode on the back of the bulb. The ERG from intact animals and enucleated eyes also give in principle corresponding results (HARTLINE, 1925).

Taking into consideration the complicated neuro-anatomical structure of the generating object, it was thought at quite an early date that the total ERG must be composed of several component potentials of different signs and amplitude, which, in varying time relations, give rise to the different phases of the retinal potential (KÜHNE and STEINER, 1880; EINTHOVEN and JOLLY 1908; PIPER, 1911 and KOHLRAUSCH, 1918). EINTHOVEN, JOLLY and PIPER studied the change in the retinogram with varying types of stimulation, and, from the phase changes obtained, they arrived at three different components. EINTHOVEN and JOLLY introduced the phase signs *a*, *b*, *c* and *d*, which have since been in general use. The important component analysis carried out by GRANIT (1933) on decerebrated cats affords greatly increased interest concerning the electrophysiology of the retina. GRANIT assumed that certain phases of the retinogram should be more sensitive than others owing to varying resistance in the source structure. It also turned out that he could selectively produce definite reversible phase changes in the ERG. In the course of the phase change, GRANIT was able to identify different components in the total effect. The different components P I, P II and P III were fixed and analysed with reference to their relation to the impulse discharge in the optic nerve.

Various vertebrates have served as objects for the numerous investigations which have since been carried out by GRANIT and his co-workers. Investigations concerning the division of the retinogram into different components and their functional relations have shown corresponding results on the whole (frog, GRANIT and RIDDELL, 1934; owl, pigeon and mouse, GRANIT, 1935; rat, CHARPENTIER, 1936). Concerning the human ERG, see pag. 70. Individual divergencies have been ascribed to anatomical or functional differences, conditions that have served to illustrate the

principal problems from different points of view. The following representation will chiefly treat facts obtained from experiments made on the frog.

Components of the Retinogram and their Relation to Impulse Discharge in the Optic Nerve.

Fig. 1 shows a schematic diagram of the components in the ERG of the dark and light adapted frog's eye (GRANIT and RIDDELL, 1934). The initial negative *a*-wave is formed by the very first, as yet uncompensated, part of the earliest appearing negative component P III. The positive component P II is superimposed on P III, and in its earlier course P II forms the contour of the *b*-wave. The slowly rising *c*-wave in the dark-adapted eye after a long period of illumination is formed by P II and the positive P I which occurs later. P III, which lies hidden after the superimposition of the positive components, rises quickly to the base line on cessation of light. P II falls slowly, and the *d*-wave, the off-effect, seems to rise schematically like an interference phenomenon between P III and P II.

P I which is only to be found in the dark adapted eye is not associated with any measurable change of impulse frequency in the optic nerve (GRANIT 1933; GRANIT and THERMAN, 1935; THERMAN, 1938). This component which is selectively enhanced by adrenaline (THERMAN 1938) seems to have something to do with the excitability in the retina, although this has not yet been explained. When using the light adapted eye the influence of P I is eliminated.

P II is the only component that is associated with impulse response in the optic nerve, and by eliminating P II the impulse discharge in the nerve disappears (GRANIT, 1933; THERMAN, 1938). P II's qualities as an excitatory component is demonstrated by its relations to the impulse picture in the optic nerve *e. g.* with variations in area, intensity and state of adaptation (*e. g.* GRANIT, 1932 and 1933; GRANIT and THERMAN, 1935).

Thus the *b*-wave formed by P II is the best electrophysiological expression for the sensitivity of the retina. It is used as a measurement of the effect from the retina when investigating the sensitivity of the retina in different states of adaptation (*e. g.* CHARPENTIER, 1936; RIGGS, 1937; WREDE, 1937; THERMAN, 1938), its relation to different wave lengths equalized with respect to energy

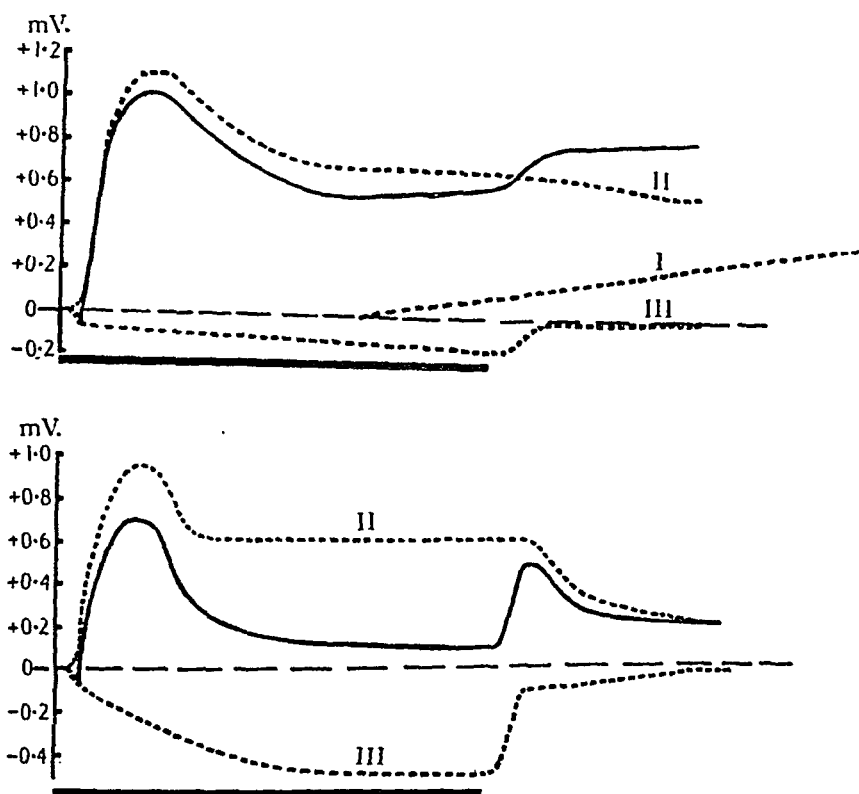


Fig. 1. Analysis of the components of frog's retinal action potential (drawn in full) in dark adaptation (upper set of curves) and light adaptation (lower set of curves). Components drawn in broken lines. Thick black line indicates a stimulus of 2 sec. duration. Explanation in text. (GRANIT and RIDDELL, *J. Physiol.*, 1934, 81: 1.).

(GRAHAM and RIGGS, 1935; GRAHAM, KEMP and RIGGS, 1935; GRANIT and MUNSTERHJELM, 1937; GRANIT and WREDE, 1937), as well as its relation to the concentration of visual purple in varying conditions (GRANIT, MUNSTERHJELM and ZEVI, 1939). The *b*-wave in the frog's retinogram often shows several smaller *b*-maxima when using localised electrodes on the retina, a phenomenon that has also been observed when using the electrodes in the ordinary position (see GRANIT, 1938). These humps in the *b*-wave will most often be attributed to simultaneous discharges from various fibre units (GRANIT and THERMAN, 1935). It has, however, also been possible to prove that similar polyphasic waves at low intensities really form variations of the retinal reaction to light (GRANIT and MUNSTERHJELM, 1937). These small *b*-maxima are independent component *b*-waves and

are explained as being retinal equivalents to fibre potentials of different functional kinds discovered by HARTLINE (*e. g.* 1938). The positive P II "is a sum total of component potentials with different adaptability and different time constants in general" (GRANIT, 1938).

When eliminating the two positive components, the illumination of the eye gives an entirely negative response, which is made up of the component P III (GRANIT, 1933; GRANIT and RIDDELL, 1934; THERMAN, 1938). The negative P III in the frog's eye, isolated by means of potassium, agrees with the first phase of the *a*-wave and the off-effect in the normal retinogram, as regards its initial fall and return to the base line in time relation and rate of rise. This agreement is also to be found with variations of the stimulus (THERMAN, 1938; GRANIT and THERMAN, see GRANIT, 1938).

Fig. 1 shows the difference in the frog's retinogram in states of dark and light adaptation. The amplitude of the *b*-wave diminishes with increasing light adaptation, the retinogram falling towards the base line, while the off-effect shows a more rapid increase to higher amplitude. The same changes with continuing light adaptation concerning the fall of the *b*-wave and the increase of the off-effect are to be obtained in preparations which are made to fall considerably below the base line after the *b*-wave (GRANIT and RIDDELL, 1934). In the case of partial or total elimination of positive P II, the off-effect can take place entirely on the negative side. Thus light adaptation favours the negative component P III.

The light adapted state is consequently characterized by the absence of the component P I and a striking change of P III, which increases in size and shows increased rapidity when returning to the base line, at the same time as the off-effect increases in amplitude. The change of P III can take place without a simultaneous change in P II (the *b*-wave).

As the off-effect was shown to be associated with a renewed intense impulse discharge in the nerve (ADRIAN and MATTHEWS, 1927 a and b; GRANIT, 1933) and P II was found to be associated with excitability; GRANIT assumed that the reappearance of the positivity of the off-effect with its accompanying impulses was to be regarded as a "post-inhibitory rebound", in consequence of P III having had an inhibitory effect on positive P II.

Just as P II has been shown to form a typical "excitatory

potential", the identification of P III as an "inhibitory potential" has gained ample experimental support. As has been pointed out, the change in the off-effect is quite opposed to that of the *b*-wave in different states of adaptation, as it increases with light adaptation. This, as well as the fact that the off-effect in the negative retinogram (P III) of the potassium-treated frog's eye copies in its rise the off-effect of the normal retinogram, made it probable that the off-effect had something to do with the component P III (GRANIT and RIDDELL, 1934; THERMAN, 1938; GRANIT, 1936 and 1938, WRIGHT and GRANIT 1938),

EINTHOVEN and JOLLY (1908) observed that the *a*-wave became more pronounced if the retinogram was recorded immediately upon a light stimulus. GRANIT and RIDDELL (1934) made this observation a basis for a quantitative investigation of the participation of the components in the off-effect. The effect of a flash, superimposed upon the off-effect on the light-adapted frog's eye, caused a strong "negative notch" in the positive off-effect. This negativity is greatest when falling on the maximum of the off-effect, and decreases at shorter and longer intervals between the cessation of the light and the following flash. The strong negativity, which implies a partial or total removal of the off-effect, passes at longer intervals in the *a*-wave of the normal retinogram and is accompanied by the positive *b*-wave. GRANIT and RIDDELL showed that the negativity produced by the superimposed light stimulus is due to a selective reactivation of the negative component P III. A flash on the maximum of the off-effect scarcely gives any "negative notch" in the dark adapted eye, and in agreement with this, the off-effect of the eye treated with potassium shows a strongly marked "negative notch" (THERMAN, 1938).

By means of this method of rendering P III selectively active during off-effect, GRANIT and THERMAN (1935) investigated the relation of the nerve impulses to the negative P III. It proved that the reactivated negativity in the retinogram corresponds to a momentary stopping of the off-impulses of the nerve, and thus a strong functional support was obtained for the identification of the P III component as an inhibitory one.

Tests made by WREDE (1937) and THERMAN (1938) show how the *b*-wave rises during dark adaptation, while the off-effect at first rises and then falls in amplitude. During the course of the dark adaptation THERMAN followed the amplitude of the off-effect and the *b*-wave at monochromatic stimuli of 0.450 μ and 0.650 μ

resp. At 0.450μ , the *b*-wave rises gradually with dark adaptation to maximum, while the off-effect only rises when the *b*-wave has reached its maximum, and then it again falls and disappears. (At 0.650μ the retinogram does not participate in these adaptive changes of the rod spectrum). According to GRANIT (1938), these observations might find their explanation in the fact that the processes releasing the on- and off-effects in the dark adapted eye divide pathways, so that the *b*-wave has a refractory action on the off-effect.

This possibility finds experimental support in important additions (GRANIT and THERMAN, 1937) to the investigations made by GRANIT and RIDDELL. The part of the off-effect, not eliminated by the superimposed flash, can be made to disappear when the off-effect occurs near a previous *b*-wave and disappears with the elimination of the component P II. This *non-inhibitable part* of the off-effect thus seems to belong to the component P II as does the *b*-wave. Whereas that part of the off-effect which reacts to superimposed light with the negative notch coinciding with the impulse inhibition in the nerve, *i. e.* the *inhibitable part*, is independent of P II. In the eye treated with potassium, the retinogram of which has been deprived of its component P II, the whole off-effect is inhibitable (THERMAN, 1938).

HARTLINE's latest investigations (1935 and 1938) of the impulses from single fibres in the optic nerve (bull-frog) show how different single fibres give different definite types of response. Some nerve fibres respond at the onset of light with an impulse outburst of high frequency followed by a constant discharge of low frequency, which ceases with the cessation of light. Other fibres discharge both at "on" and "off". Finally a third group gives a discharge only at the cessation of light, but this is of a strong and lasting character. The discharges of this latter group are checked by re-illumination.

Thus, it seems (GRANIT and THERMAN, 1937), as if the non-inhibitable part of the off-effect due to positive P II would form a retinal equivalent to the effect from that type of fibre which, according to HARTLINE, discharges both at the onset as well as at the cessation of light. The inhibitable part of the retinal off-effect is, on the other hand, related to the elements, the off-discharge of which is inhibited by reillumination.

Veratrine, however, brings the off-effect to a state of complete inhibitability without the *b*- and *d*-waves undergoing any change

of amplitude (THERMAN, 1938). On this ground THERMAN assumes that the distinction between the two components of the off-effect is not absolute, while the veratrine effect indicates that the negative notch due to P III forms a significant factor for both components.

Nature and Origin of the Retinal Components.

Ever since GRANIT's first work in 1933, the difference between the components of the retinogram as well as their correlation to opposite functional qualities have received many criteria. The above-mentioned tests show how two potentials of opposite signs are produced in the retina when illuminated and how these potentials are associated with excitability and inhibition respectively.

THERMAN's observations on the effect of different nerve poisons on the excitability of the retina (1938) confirm still more definitely GRANIT's analysis of the components as well as the relation of P II and P III to excitability and inhibition.

P II seems to be necessary to enable an impulse discharge to follow in the nerve, although in certain cases (*e. g.* in the initial state of potassium influence) P II can exist without impulse discharge following.

P III has been shown to stand in relation to active inhibition, which is excellently demonstrated by the association of the retinal negativity with the interruption of existing off-impulses in the nerve.

The opposite signs of the components in relation to the functional states associated with them are of great interest from a general neurophysiological point of view. The words positive and negative refer to the usual manner of placing the electrodes. Tests on an isolated retina show that in reality P II implies the negativity and P III the positivity of the receptorial side of the retina relative to the ganglion layer (WRIGHT and GRANIT, 1938; GRANIT and THERMAN, 1938). The reversal of the signs may be explained by the anatomical inversion of the vertebrate retina (*e. g.* WRIGHT and GRANIT, 1938).

As regards signs and accompanying states of excitability, the component P II generally agrees with the slow negative potential in other ganglion structures. P II has thus been compared with the slow negative potential in the spinal cord (GASSER and GRAHAM, 1933) and in the superior cervical ganglion (ECCLES, 1935 a

and b). On an analogy the component P III, which is associated with inhibition, has been generally compared with the slow positive potential in the same nervous structures (GRANIT, 1933; GRANIT and THERMAN, 1935).

As a basis for extensive investigations of the relation of the retinal excitability to different nerve poisons, THERMAN (1938) proved the tenability of a close comparison of the components P II and P III with the after-potentials in peripheral nerves and slow ganglion potentials elsewhere.

THERMAN's observations on the reaction of P II and P III to the different nerve poisons (potassium, calcium, veratrine, and strychnine) do not show sufficiently satisfactory agreement with the results obtained on peripheral nerves (GRAHAM, 1933; LEHMANN, 1937; GRAHAM and GASSER, 1931; GRAHAM 1930) to identify the retinal components with the after-potentials in the peripheral nerves.

Owing to the fact that nerve poisons have hitherto been tested to a very limited extent on slow ganglion potentials (LEHMANN, 1937; ECCLES, 1935 b), THERMAN has delayed coming to any conclusion concerning the comparison of the retinal components with other slow ganglion potentials.

In order to illustrate the causal relation between the retinal potential and the impulses of the optic nerve, and in order to investigate whether P II and P III are true opposite potentials, GRANIT and HELME (1939) tested the influence of electrotonic states on retinal excitability.

Retinal inside cathode increased the on- and off-effects, both in the retina and the nerve, while the retinal inside anode produced a decreasing effect. Polarizing current has the same influence on the negative component P III (obtained by treating the eye with potassium). The fact that P II and P III seem to be similarly influenced by a polarizing current appears to indicate, according to the authors, that the components really are potentials of opposite signs. This result makes it scarcely probable that their opposite signs would be due to a different orientation of the respective source structures in relation to the electrodes.

The experiments with polarization show how the retinal excitability is influenced by electrotonic states. The authors point out the possibility of the fact that the spread of excitability in the retina might be of an electrotonic kind, a possibility even expressed by GRANIT and THERMAN (1938).

Proceeding from the argument that ECCLES (1935 b) used as a basis for pointing out the correspondence of the P and N processes with cat- and anelectrotonus respectively, THERMAN (1938) illustrated the influence of poisons on the different states of catelectrotonus and anelectrotonus in the retina, if P III were to be regarded as a state of anelectrotonus.

Investigations by GRANIT (1933), GRANIT and THERMAN (1935) and THERMAN (1938) show that the ERG is not made up of summed impulse potentials. The fact that synchronized impulse potentials may be superimposed on the retinogram, as well as assumptions for this have been previously discussed.

HOLMGREN (1880) attributed *a*, *b*, and *d* to the retina, and KÜHNE and STEINER (1880) showed by means of experiments on the isolated retina the correctness of this statement. The latter authors ascribed the whole of the retinogram to the basal ends of the sensory cells, while GARTEN (1907) considered the outer part of the receptor cells to be the source of the ERG.

An increase in the intensity of excitability causes a shortening of the latency of the retinogram (EINTHOVEN and JOLLY, 1908; ISHIHARA, 1906; ADRIAN and MATTHEWS, 1927 a; GRANIT, 1932) and the optic potentials (ADRIAN and MATTHEWS 1927 a and b). A shortening of the latency is obtained also with an increase of the illuminated area (ISHIHARA, 1906; ADRIAN and MATTHEWS, 1927 a and b; GRANIT, 1933). As has already been said, ADRIAN's and MATTHEW's investigations show that latency shortening with an increasing illuminated area is due to synaptic interaction. They show, too, that the interval from the beginning of the *a*-wave to the onset of the nerve impulses is constant. These facts seem to imply a localization of the retinogram at a point situated centrally to the lateral connections of the retina (GRANIT, 1933). Moreover, the fact that the ERG shows typical interaction phenomenon touching both P II and P III has also been taken as a proof of a synaptic or post-synaptic localization of the retinogram (GRANIT and THERMAN, 1935).

The grasshopper's eye, which has two layers of neuro-sensory cells, shows a complete retinogram with all the *a*, *b*, *c*, and *d* waves (CRESCITELLI and JAHN, 1939) resembling the ERG of the vertebrate eye. The eye of LIMULUS with only one layer gives on the other hand a monophasic retinogram (HARTLINE, 1928; HARTLINE and GRAHAM, 1932). Thus, one component associated

with impulse discharge in the nerve agrees with regard to the electrical sign with P II. This, as well as the fact that the receptors throughout the phylogenesis may be regarded as the same kind of cells (KAPPERS, HUBER and CROSSBY, 1936) makes it difficult, according to GRANIT and HELME (1939), not to correlate at least one of the components of the vertebrate eye with the only component of the eye of *Limulus*.

The above conclusions do not altogether support these last mentioned observations, which seem rather to make it probable that at least one of the components is to be assigned to the receptor layer (GRANIT and HELME, 1939).

GRANIT and ECCLES as well as GRANIT and HELME (see GRANIT and HELME, 1939) also tested the effect of antidromic volleys on the optic nerve but received no response from the retina, which made them conclude that the ERG, or any fraction of it, can hardly be related to the ganglion cell layer. The authors are also under the impression that the polarizing effect on the retinogram is not due to the influence of polarization on this cell layer.

It has also been mentioned that GRANIT and THERMAN (1938) and GRANIT and HELME (1939) suggested the possibility that the components of the retinogram or their fractions might show the quality of electrotonic spreading. According to the latter authors, a similar quality might be able to explain the somewhat contradictory observations concerning the difficult problem of the origin of retinal potential.

Slow Potentials in the Roots of the Spinal Cord.

In experiments on the cat and the frog, BARRON and MATTHEWS (1938) analysed the effect in the dorsal and ventral roots of the spinal cord at electric (via the dorsal roots) and natural stimulation (brief sensory stimuli). They found both in the dorsal and ventral roots slow potential changes (cp. GOTCH and HORSLEY, 1891; UMRATH, 1933; ECCLES and PRITCHARD, 1937; BONNET and BREMER, 1938) which are not caused by summed nerve impulses but seem to be due to spread by electrotonus of potential changes occurring in the grey matter of the spinal cord.

These slow potentials showed, quite independently of the nature of stimulation, always the same sign with the negativity in the electrode placed nearest the spinal cord. The slow potential of the dorsal roots showed signs of spatial and temporal summation

as well as occlusion. The slow potential of the ventral roots precedes the impulse discharge, and are checked in their development by such stimuli as inhibit impulse discharge.

The authors give the following schematic summing-up of the experimental results which are also discussed in relation to the membrane theory of nervous action:

"An afferent volley in the dorsal root gives rise to depolarization at the primary terminations in the spinal cord, observed as dorsal root electrotonus. This sets up impulses in the internuncial neurones. These impulses produce depolarization of the motor neurones, which sets up an impulse discharge in the ventral roots. The depolarization of the motor neurones is observed as the ventral root electrotonus."

Author's Investigations.

The preceding pages give a brief account of the successful analysis of the electrophysiology of the retina which has been carried out by GRANIT and his co-workers. The points of view associated with the division of the retinogram into components have been presented, which components have been ascribed to different functional states. It will be understood how interest has concentrated *inter alia* around the difficult problems concerning the origin and kind of slow potentials appearing in the retina, problems requiring considerable experimental work undertaken from various angles in order to bring about a solution.

GRANIT and THERMAN (1938) and GRANIT and HELME (1939) stated that it is possible that the slow potentials of the retina may show electrotonic spread. Such a proof would give a new experimental basis for the study of the spread of excitability in the retina.

BARRON's and MATTHEWS' investigations (1938) seem to show that the slow potentials which they registered in the roots of the spinal cord are the result of electrotonic spread of the electric changes taking place in the grey matter of the spinal cord.

These discoveries invite to a closer analysis of the effect of natural stimulation of the retina in the optic nerve. We here meet with complications in the combined potential picture, which is produced by the summed more or less synchronised fibre impulses. The off-effect, however, gives us an accessible analysis basis on

account of the off-impulse in a state of discharge being effectively brought to a stop by superimposed light on the off-effect (GRANIT and THERMAN, 1935).

By making use of the technique with superimposed light on the off-effect, the effect from the optic nerve has been investigated and compared with the potential picture of the retina obtained under similar conditions.

The investigations thus made are intended to show whether a slow potential can be identified in the optic nerve when the retina is stimulated.

Methods.

Apparatus.

In order to register the action potentials from the retina and the optic nerve a Loewe cathode ray tube with two beams was used (type KSH 20/2).

In most tests the oscillograph was used in conjunction with a 4-stage push-pull coupled amplifier (C. v. Sivers, Svenska Radioaktiebolaget) of a type resembling in principle the one employed by GRANIT and THERMAN (1935) and THERMAN (1938). For recording both the retinogram as well as the action potentials from the optic nerve this amplifier was used direct-coupled. This always gave a good and steady base line and also satisfactory proportional amplification. The absence of drift at the base line was controlled in the case of each registration. Calibrations 30 and 100 μ V at maximum amplification, see fig. 2 A and B.

Simultaneous records from the retina and from the nerve were taken by means of the two balanced condenser coupled amplifiers used by GRANIT and HELME (1939) in conjunction with the double cathode ray oscillograph. Simultaneous calibrations with 100 μ V are recorded in fig 2 C.

Time was recorded in $\frac{1}{6}$ sec. throughout by means of a Jacquet time marker.

"White light" was used as stimulus from a 100-watt Osram projection lamp placed at a distance of about 50 cm. from the preparation in a shaded lamp holder with a circular hole for the emerging ray of light. The outgoing beam passed a system of lenses and prisms by the help of which the light was focussed and brought to fall on the prepared eye. For the intensity variations Wratten neutral tint filters were used with standardized density. In all the following illustrated tests full intensity has been used. An iris diaphragm in the beam allowed variations of the illuminated area.

A Compur shutter placed in the beam was used for the approximately constant standard exposures, and this was manipulated by hand. The Compur shutter was used in order to obtain longer gaps of darkness in the continued illumination. The shorter gaps of darkness were obtained

by sliding a small screen quickly through the ray of light. A water filter was also placed in the beam.

A narrow ray was diverted by means of a mirror placed between the shutter and the preparation. This was directed to the camera by means of mirrors in order to give direct marking of the light stimuli on the bromide paper.

All records were made on bromide paper in a camera which was driven by an electric motor giving no disturbances.

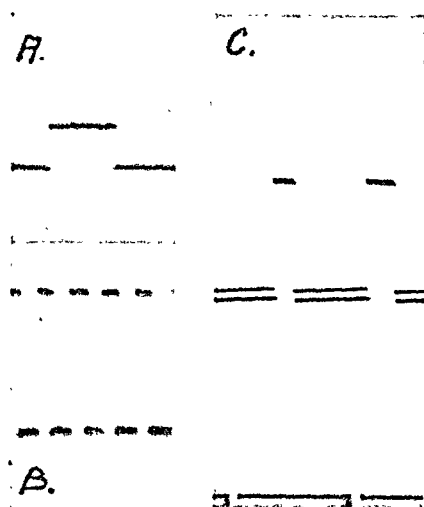


Fig. 2. A and B, calibrations with 30 and 100 μ V resp. of direct-coupled amplifier. C, simultaneous calibrations with 100 μ V of condenser coupled amplifiers. Time in $\frac{1}{8}$ sec.

Preparations.

The retina and nerve preparations used were chiefly from *Rana temporaria*, *Rana esculenta* being used only in a few cases.

The preparations were carried out according to instructions given by GRANIT and his co-workers (GRANIT and RIDDELL, 1934; GRANIT and THERMAN, 1935 and 1937; THERMAN, 1938) in order to avoid the complicating factors to which they called attention.

Eyes intended only for the ERG were enucleated and opened carefully, the cornea, the iris and the outer part of the vitreous body being removed. Prepared eyes with remaining parts of the vitreous body to protect the retina from drying thus gave constant deflections for upwards of 40 mins. (cp. THERMAN, 1938). A steady base line or slow drift in the main direction of the retinogram indicates, as THERMAN points out, a good and durable preparation. Decreasing amplitude in the ERG and opposite drift in the base line was regarded as a sign of deterioration. Records were only taken as long as the preparation gave constant deflections, and the experiments seldom lasted for more than 30—40 mins.

The enucleated and opened bulb was resting on cotton wool soaked in Ringer solution and was placed on a special little ebonite holder.

The preparation was made somewhat differently for the simultaneous recording of the action potentials from the optic nerve with the ERG. The eye was treated in the same manner but remained in connection with the nerve. The nerve was dissected along the whole of its length and was kept connected with the optic decussation. The whole preparation thus obtained was placed on the holder. The eye was then lifted up to rest in a thin isolated metal ring so that the nerve, which is 5—6 mm. long, stood like a thin stalk and in connection with the decussation on the brain stem. This was kept either in situ or else a small part round the decussation of the nerve was prepared in order to serve as a foundation for the one electrode. The preparation was moistened with Ringer solution before each experiment, and gave constant deflections from both retina and nerve for about 30 mins.

Thin silver chlorinated pins covered with cotton wool which was drawn out to a thin point were used as electrodes. The ERG was taken from the cut retinal edge and back of the bulb. To record the action potentials from the nerve, either both the electrodes were placed on the nerve, or else one electrode on the nerve and the other on the attached part of the brain. Special variations concerning the position of the electrodes will be described below.

During the experiment the preparation lay on the holder which was placed on a firm stand and the electrode holder was also fixed here. The stand was put inside an electrically shielded box, which was connected to the earth by means of the shielding of the cables. The preparation box was furnished with a circular hole for the incoming light.

The experiments were carried out during all the four seasons of the year, and in order to obtain uniform conditions in which to perform the experiments the animals were kept in the dark and in an ordinary room temperature for 12 hours previous to the experiment (GRANIT and THERMAN, 1937; THERMAN, 1938). Only light adapted eyes were used, and the preparation was consequently carried out in good illumination. When the preparation had been fixed on the holder, it was left to rest for about 5 mins., during which time it was illuminated by the test-light, which was also used as adapting light. The same state of light adaptation was obtained by letting the light shine during the whole experiment. When the constancy in the off-effect had been tested a few times at regular intervals of 1 sec., the registration of the effect followed at gaps of darkness of varying length in the adapting light, the gaps being given at regular intervals of 1 sec. It is not necessary to keep the eye in any particular state of adaptation, something that GRANIT and THERMAN (1937) pointed out. It is only important for the adapting light to be kept within a range of intensity in which the off-effect does not fluctuate. As will be seen from the diagrams, the off-effect in these tests remained constant both in the retina and the nerve. In these conditions the off-effect of the retina was about 0.5—0.6 mV. Those preparations which showed from the first great variations in the amplitude were not used.

Results.

Introductory Experiments demonstrating Excitation and Inhibition in the Retina and the Optic Nerve.

Fig. 3 shows a series of tests in which the retinograms were taken from a light adapted frog's eye at different gaps of darkness in the adapting light, *i. e.* the adapting light being superimposed

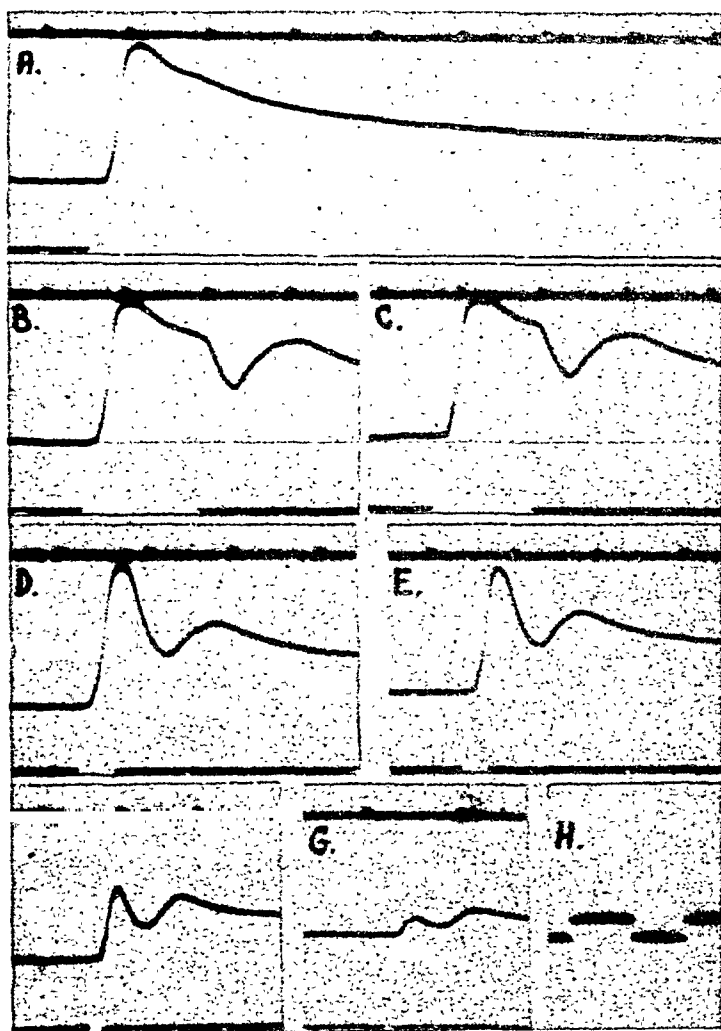


Fig. 3. Oscillograph records (direct-coupled amplifier) of retinal responses of light adapted frog's eye. A, normal off-effect. B—G show the effect of re-illumination during the off-effect at varying intervals after cessation of light. H, calibration 100 μ V. Time in $1/8$ sec.

on the off-effect at different intervals. The records A, B, and C show that the off-effect has the same amplitude through the series of tests, which indicates that the conditions have been identical and favourable.

The shorter the gap of darkness the greater the negativity produced by the superimposed light. The negative notch is greatest on the maximum of the off-effect (fig. 3 D) and afterwards diminishes when the gaps of darkness are made shorter.

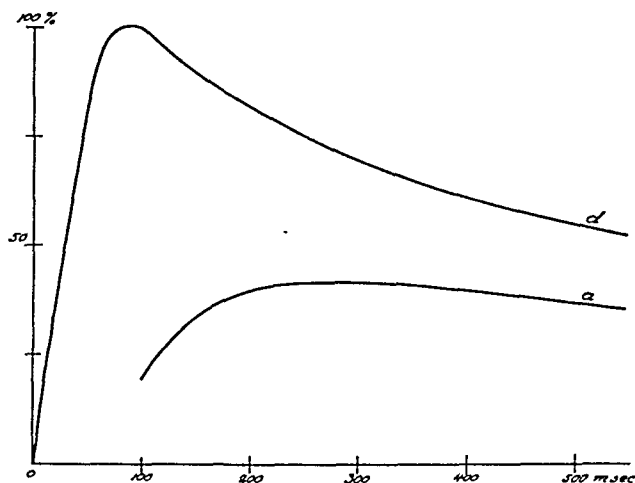


Fig. 4. Diagram representing data obtained from the retina in a typical experiment (fig. 3) with re-illumination during the off-effect after varying gaps of darkness. Curve *d* is the average retinal off-effect. Curve *a* gives the level of negative dips occurring along the retinal off-effect. The amplitudes are given in percentages of the maximum amplitude of the normal off-effect. Time in msec. with zero time at the beginning of the off-effect.

In the earliest stage of the off-effect, the superimposed light is not powerful enough to add anything further to the negative component (fig. 3 F and G), a certain recovery from the negativity being required to bring about this, which recovery is represented by the off-effect (GRANIT and RIDDELL, 1934) or a part of it (GRANIT and THERMAN, 1937). The negative notch thus increases with increasing recovery (fig. 3 C—D), which occurs with great rapidity.

The following *b*-wave, which is extremely slight in short gaps of darkness, increases with increasing intervals. According to GRANIT and RIDDELL, its amplitude increase illustrates the recovery of the component P II, which thus takes place considerably more slowly than that of component P III.

According to GRANIT and THERMAN (1937), however, the non-inhibitable part of the off-effect should belong to P II, as they

maintain that the diminished amplitude of the *b*-wave at short gaps of darkness is partly due to its falling within the refractory period of that part of the off-effect belonging to P II, and partly to P I II showing a distinctly slow recovery at the cessation of the illumination.

The diagram in fig. 4 classifies the previously illustrated series of experiments in a manner similar to that adopted by GRANIT and RIDDELL. The *d*-curve represents the average off-effect, while *a* shows the level of negative dips occurring along the off-effect. The time is given in milliseconds from the beginning of the off-effect. In order to be able to make a comparison of the data from the different preparations of the retina and to compare the general course of the curves when testing the retina and the nerve, the amplitudes are most suitably indicated in percentages of the maximum amplitude of the off-effect.

In the figure *a* represents that part of the whole of the off-effect (*d*) that is not removed by the negativity produced, *i. e.* the non-inhabitable part, the difference between *a* and *d* forming the inhabitable part. According to GRANIT and THERMAN, the former should be thus connected with P II, the latter making up the component P III.

Figs. 5 and 6 give some simultaneous records of the ERG and the impulse discharge in the optic nerve. Figs. 5 A and 6 A show the normal off-effect with well marked synchronised impulse discharge in the nerve. Records B and C in both figures indicate the obvious effect discovered by GRANIT and THERMAN (1935) of a superimposed light stimulus on the off-effect.

The already described retinal negativity appears with re-illumination, and parallel with this the nerve impulses are suddenly inhibited, thus causing a short impulse gap. Not until after this does the fresh impulse discharge follow. As GRANIT and THERMAN point out, the latency of the post-inhibitory excitation (the distance between the on-set of the fresh light and the beginning of the following on-impulses) increases with diminishing gaps of darkness, see figs. 5 B, 6 B and C. This period includes both the duration of inhibition and the latency of the excitation. It is difficult to decide how the prolongation of the whole period is divided between them (GRANIT and THERMAN, 1935).

The figures also illustrate the fact indicated by ADRIAN and MATTHEWS (1927 a) that the on-impulses in the optic nerve begin during the *a*-wave (figs. 5 and 6 B and C). The exact relation

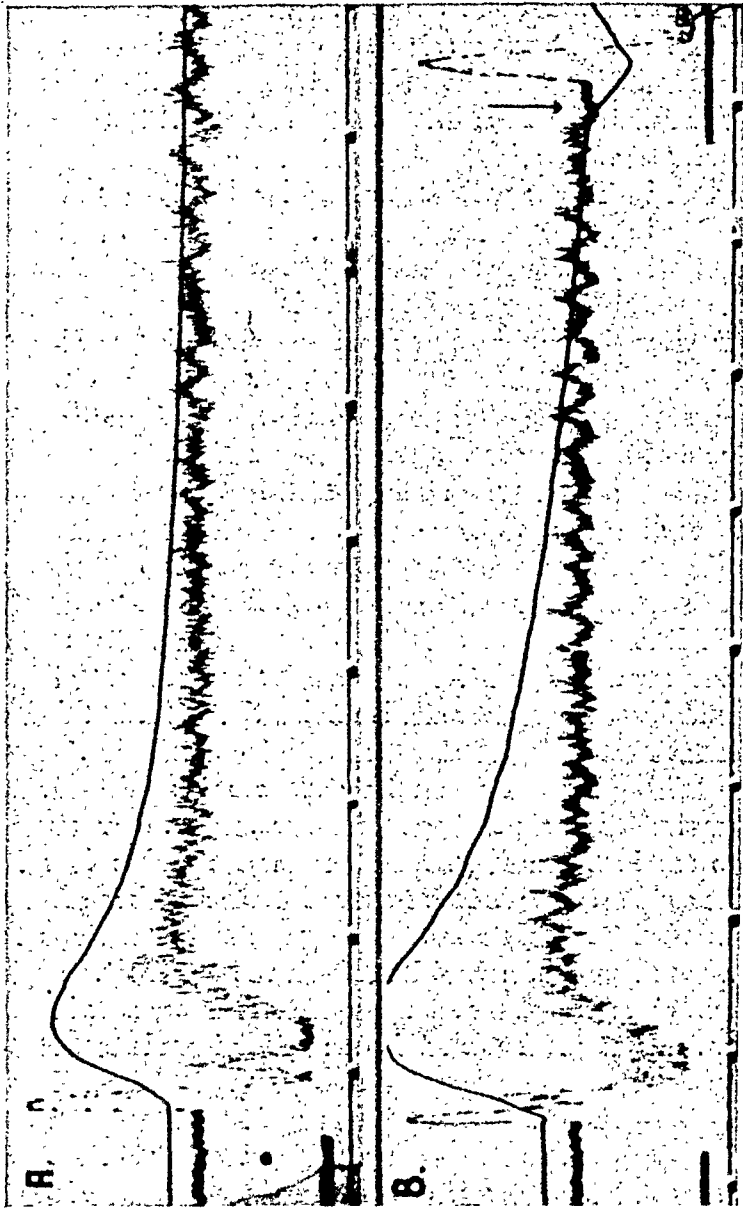


Fig. 5. Simultaneous oscillograph records (condenser coupled amplifiers) from the retina (upper curve) and the nerve (lower curve) of light adapted frog. A, normal off-response. B shows the effect of re-illumination after a long gap of darkness. Note retinal negativity and cessation of impulse discharge in the optic nerve (at arrow). Time in $\frac{1}{16}$ sec.

between the appearances of the retinal excitatory component P II and the impulse discharge in the nerve (discussed as synaptic delay) is equally difficult to state, the exact moment for the appearance of the *b*-wave not having been determined, as the deepest point of the negativity represents only the time when the positive component P II overcomes the negative component P III (GRANIT, 1933; GRANIT and THERMAN, 1935).

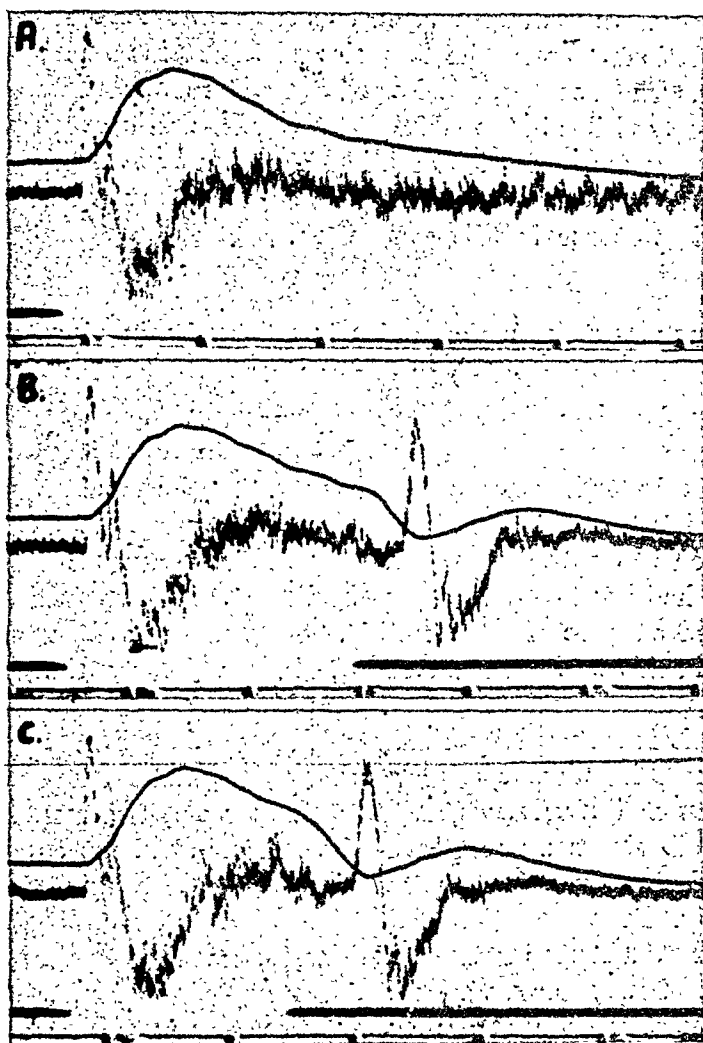


Fig. 6. Same experiment as in fig. 5 but shorter gaps of darkness.

According to GRANIT and HELME (1939), a slight retinal positivity starts at off a few milliseconds previous to the discharge in the nerve, after which the steep off rise follows a few milliseconds after the nerve impulse. This last mentioned fact is fully clear in figs. 5 and 6. The slight early positivity which, according to the authors, might possibly represent the commencement of the return of the component P III to the base line, is scarcely visible in these records, as it requires higher amplification.

These points show the difficulty of coming to any conclusion

with regard to the internal relations of the processes by means of the relation between the latencies of the retina and the nerve effects. The observations have been put forward because they are clearly visible in the different illustrations. The exact latencies will not be considered in detail in future, partly on account of the just mentioned reason, and partly because they are of subordinate importance in this connection.

**Responses from Different Parts of the Optic Nerve when
Light is superimposed on the Off-effect.**

Records taken from the nerve under the same experimental conditions, though with the use of direct-coupled amplification, present themselves as illustrated in fig. 7. Here, as in the experiment illustrated in figs. 5 and 6, the one electrode was placed right on the optic nerve, while the other was placed on a part which had been cut out of the brain substance, the nerve being pinched close to the decussation. Upward deviation means the same in this case as henceforward, *viz.* negativity in the electrode nearest the retina. The effect shows a rapid rise at on and off, and this is followed by a slower fall to the base line. Synchronized fibre impulses appear on the main effect chiefly at off (cp. ADRIAN and MATTHEWS, 1927 a; GRANIT and THERMAN, 1935). Fig. 7 D shows a normal on-effect, recorded about one second after the previous off after the cessation of the lasting off impulses (cp. fig. 5).

On record A the impulse inhibition makes itself apparent at the arrow. The clearly visible synchronized fibre impulses cease, and the curve now shows a slight tendency to fall towards the base line, while B and C show the effect at considerably shorter gaps of darkness. In the two latter cases the disappearance of the impulses is accompanied by a steep fall of the off-effect.

The inhibition of the impulses, however, never produces a fall of the curve right down to the base line, and instead the lowest point previous to the post-inhibitory discharge generally remains at varying gaps of darkness as the negative dip in the retinal records.

The following on-effect amplitude decreases with diminished gaps of darkness.

Fig. 8 illustrates the series of experiments, in the same manner, as was used for the ERG in the diagram in fig. 4, d_1 , giving

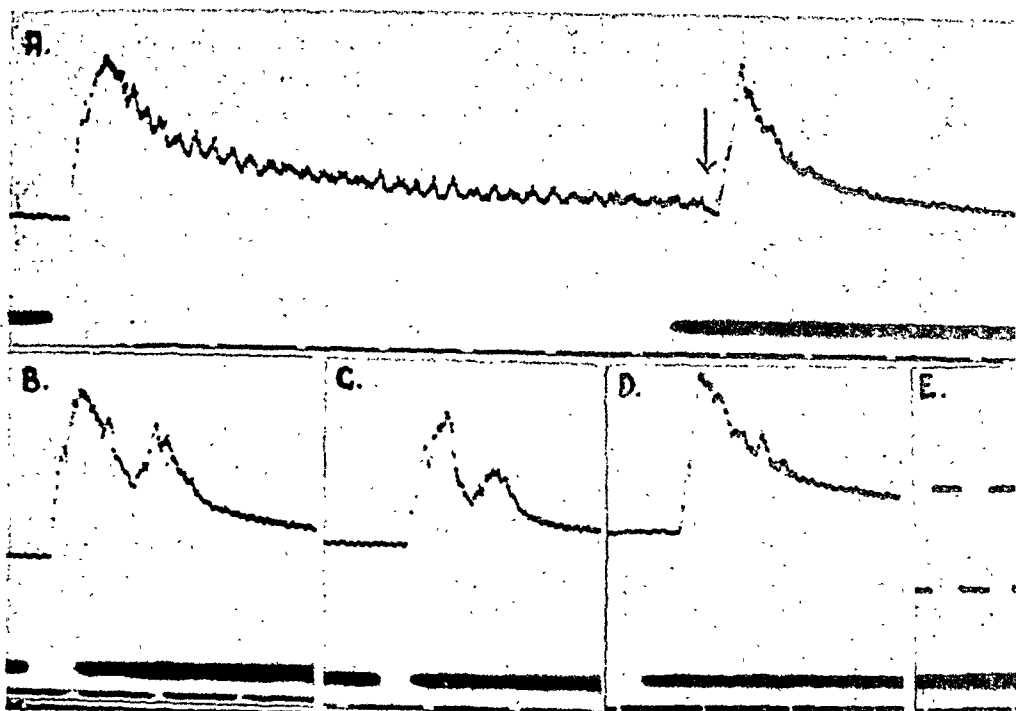


Fig. 7. Oscillograph records (direct-coupled amplifier) taken from the optic nerve (see text) of light adapted frog. A—C show the effect of re-illumination during the off-effect at varying intervals after cessation of light. Note the synchronized wavelets in A, which disappear with re-illumination (at arrow). D, normal on-effect. E, calibration 300 μ V. Time in $\frac{1}{5}$ sec.

the average off-effect in the nerve, and a_1 representing the lowest point reached at different gaps of darkness.

By placing both electrodes on the nerve, a similar result is obtained in principle. Fig. 9 illustrates an experiment where the electrodes have been placed about 1 cm. apart, making a distance of $\frac{1}{3}$ of the length of the nerve from the retina. The spike potentials are here more clearly visible (in fig. 9 A synchronization) on the curve falling towards the base line. B—F clearly illustrate the inhibition the impulses ceasing simultaneously with the fall of the curve. The increase of the period of inhibition stands out especially with the diminishing gap of darkness. In fig. 9 the nerve has been pinched close to the retina but still remains connected. No effect is visible.

As will be seen from the illustration, records with the electrodes in these positions show some small spike potentials previous to the appearance of the rapid and great deflection.

Assuming that the total effect is formed exclusively by the summed rapid action potentials, the curve d_1 in fig. 8 would approximately illustrate an "integrative recording" and form the frequency-time curve of the nervous discharge. The frequency of the nerve impulses rises rapidly to an early maximum both at "off" as well as at "on", after which the frequency gradually decreases (ADRIAN and MATTHEWS, 1927 a; GRANIT and THERMAN, 1935). The initial outburst of impulses is represented in the above figures by the steep upward initial deflection. In the period

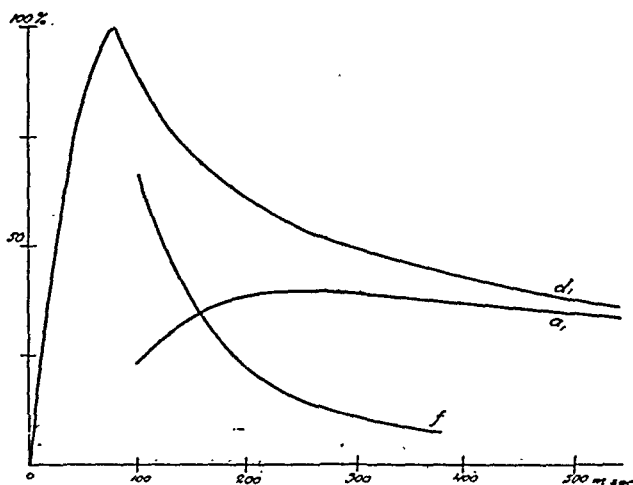


Fig. 8. Diagram representing data obtained from the optic nerve in a typical experiment (fig. 7) with re-illumination during the off-effect after varying gaps of darkness. Curve d_1 is the average off-effect. Curve a_1 gives the level of negativity occurring along the off-effect. Curve f represents the difference between d_1 and a_1 . The amplitudes are given in percentages of the maximum amplitude of the uninterrupted off-effect. Time in msec. with zero time at the beginning of the off-effect.

where the frequency is greatest inhibition will be most apparent (see e. g. 7 B). When the inhibition of impulses appears, the effect does not fall, however, to the base line, although the inhibition of the visible impulses is so striking that the contour in the period of inhibition is as free from the impulses as the undisturbed base line before the beginning of the off-effect (e. g. fig. 9 C—F).

As will be seen, it is a considerable part of the total "integratively" registered off-effect of the nerve (represented by a_1 , in fig. 8) that remains when the second light is superimposed.

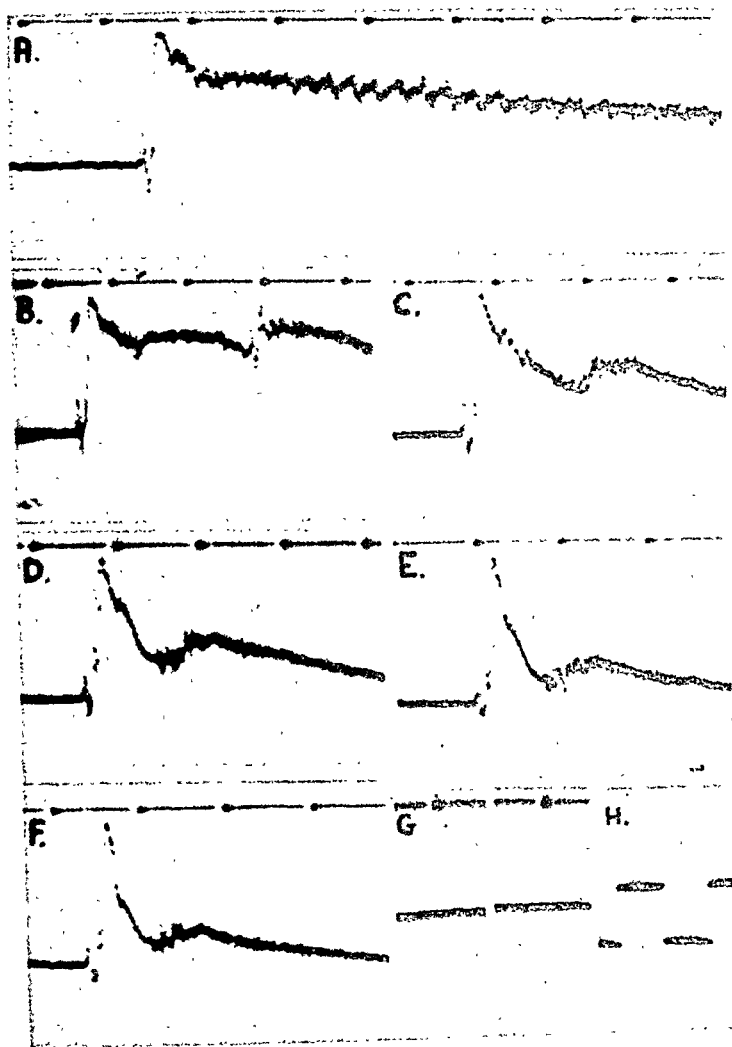


Fig. 9. Oscillograph records (direct-coupled amplifier) taken from the optic nerve of light adapted frog, with both electrodes on the living nerve (see text). A, normal off-effect. B—F show the effect of re-illumination during the off-effect at varying intervals after cessation of light. G is obtained after pinching the nerve between the retina and the electrodes. H, calibration 100 μ V. Time in $\frac{1}{8}$ sec.

Some qualities of the remaining potential have been more closely analysed in the series of experiments in which the effect has been taken from different parts of the optic nerve.

One of the electrodes was placed 1.5 mm proximally to the retina, after which the same electrode was placed 1.5 mm distally to the decussation. The other electrode remained indifferent in both cases, as it was applied to the under side of the brain, the

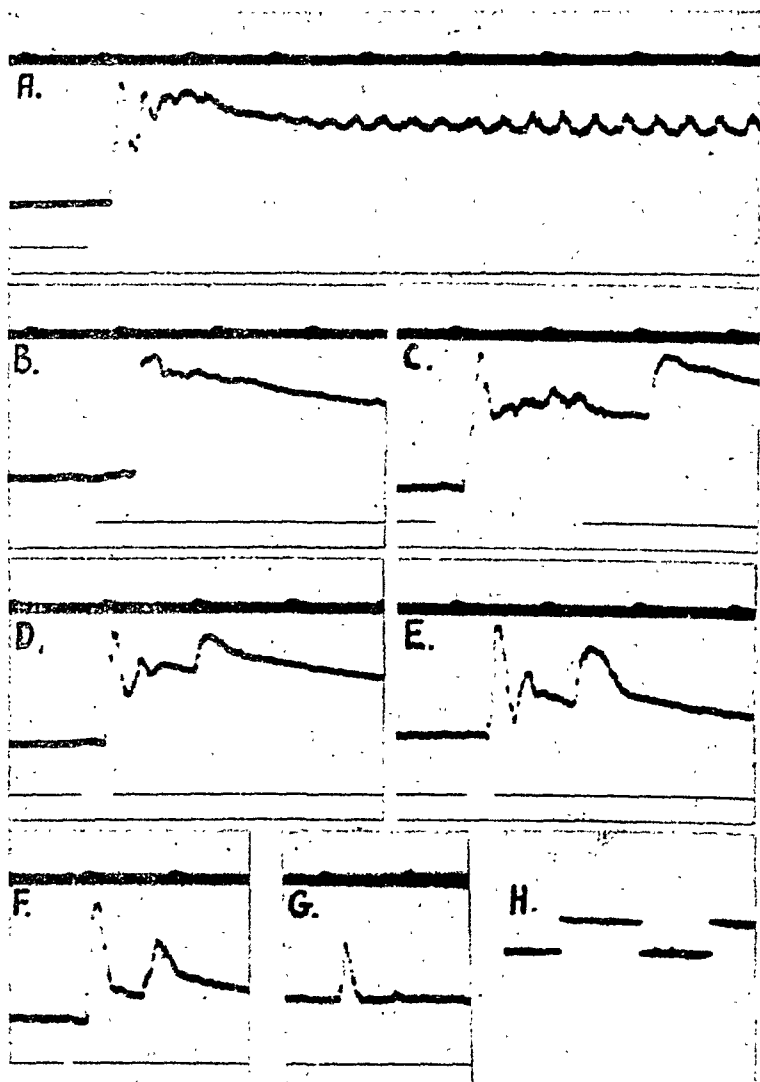


Fig. 10. Oscillograph records (direct-coupled amplifier) taken from the optic nerve of light adapted frog. "Distal recording" (see text). A, normal off-effect. B, normal on-effect. C—G, show the effect of re-illumination during the off-effect at varying intervals after cessation of light. H, calibration $100 \mu\text{V}$. Time in $1/5$ sec.

nerve being pinched close to the decussation. In each experiment the active electrode was then again placed in its original position and the effect controlled, after which registration took place when the nerve had been pinched between the retina and the active electrode. Finally both electrodes were placed on the retina in order to control the ERG.

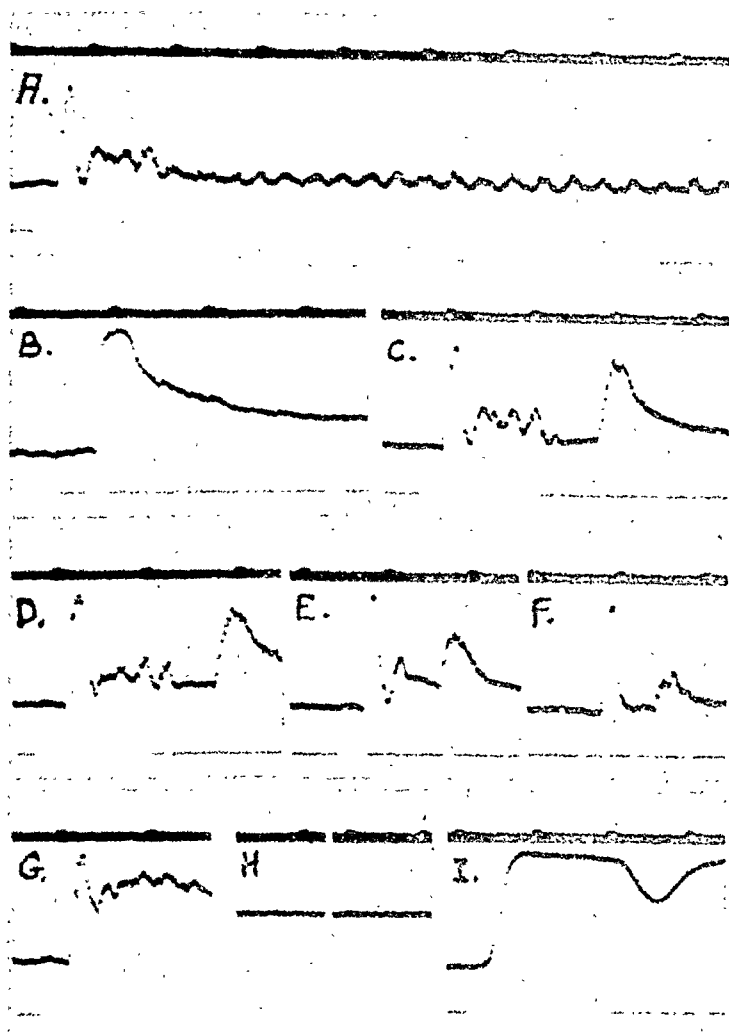


Fig. 11. The same experiment as in fig. 10 but in records A—F "proximal recording" (see text). A, normal off-effect. B, normal on-effect. C—F show the effect of re-illumination during the off-effect at varying intervals after cessation of light. G, "distal recording" (see text), normal off-effect. H, "distal recording" after pinching the nerve between retina and electrode. I, retinal response. Time in $\frac{1}{8}$ sec.

Figs. 10 and 11 show typical records from such a series of experiments. Recording with the active electrode near the retina (distal recording) gives the result illustrated in fig. 10. As will be seen, the preparation gave an off-effect largely resembling that in fig. 7. A steep rising peak-like deflection changes into the slow fall of the effect to the base line. The impulses show a syn-

chronization to units of a frequency of about 15 pr. second. The inhibition finds clear expression when the synchronized impulses cease. No definite deviation towards the base-line of the general contour of the off-effect is to be seen at the gaps of darkness when the impulse is inhibited. At short gaps (fig. 10 E and F) the new on-effect, on the other hand, rises from a lower level than that of the "normal" off-effect. After a very short gap of darkness as seen in fig. 10 G, the following on-effect rises scarcely above the base line.

The curves recorded in fig. 11 appear entirely different, the electrodes having been placed on the proximal part of the nerve (proximal recording). This striking difference is caused by the off-effect rapidly falling to the base line immediately after the initial deflection (fig. 11 A). The initial effect shows, however, the same amplitude in this case as in the above. The on-effect (cp. fig. 10 B and 11 B) appears to undergo the same change. The changes with increasing gaps of the inhibition of impulses and of the superimposed on-effect are the same in both cases. These phenomena go on, however, quite near (fig. 11 E) or on the base line (fig. 11 C and D) in proximal recording.

The active electrode is then again moved to its first position near the retina. Fig. 11 G shows that the effect illustrated in fig. 10 remains unchanged.

If the nerve is pinched between the retina and the active electrode which retains its latter position, stimulation of the retina gives no effect (fig. 11 H). Finally fig. 11 I shows the retina still giving a perfect response, as regards both the positive and negative components.

Altogether some 70 experiments have been carried out and have covered the parts illustrated in figs. 3, 7, 9, 10 and 11. In some cases (see below) the preparations have lasted so well that complete series of experiments have been carried out both on the retina and the nerve (distal as well as proximal recording) on the same object. At each recording 10—20 records have been made at varying gaps of darkness.

As regards the main principles discussed above, the experiments have given uniform results. Naturally the effect from the nerve may vary somewhat in appearance owing to the complexity of its composition. The impulses may be more or less visible on the main effect, the tendency to synchronization may vary, and the return of the off-effect to the base line may show trifling varia-

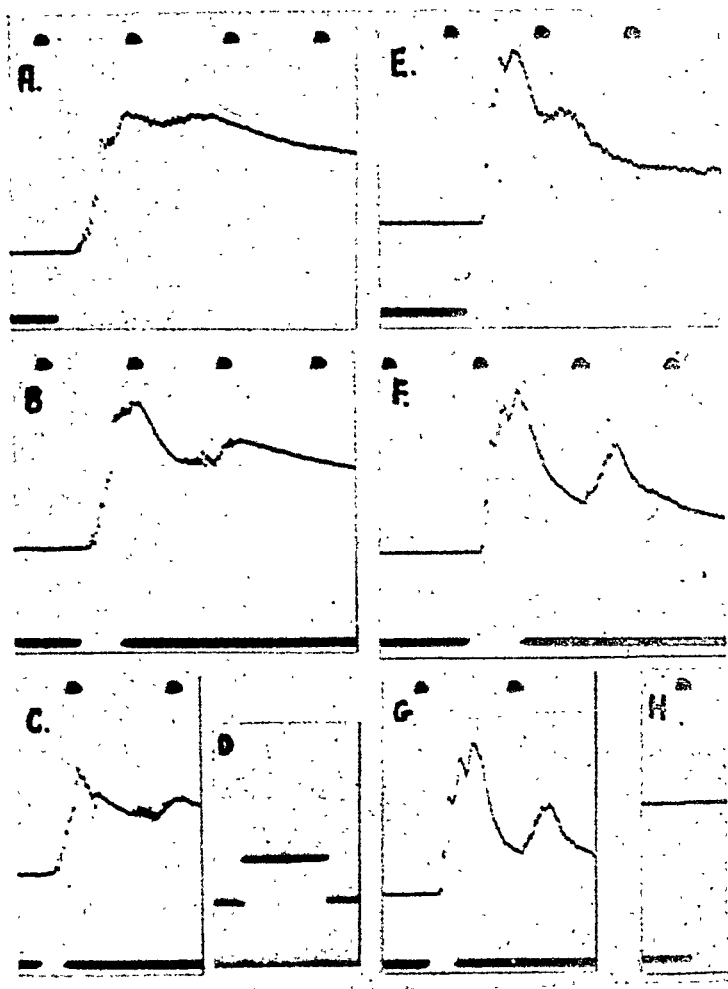


Fig. 12. Oscillograph records (direct-coupled amplifier) taken from the optic nerve of light adapted frog. A—C, "distal recording". E—G, "proximal recording" (see text). D, calibration $100 \mu V$. H, "distal recording" after pinching the nerve between retina and electrodes. Time in $\frac{1}{5}$ sec.

tions. Records in fig. 12 from an experiment with distal and proximal recording show slight differences, as regards the phenomenon mentioned above, from the curves illustrated in figs. 10 and 11. The variations may be said to lie between these two types. Fig. 12, however, illustrates the uniformity concerning the principles discussed in the foregoing pages.

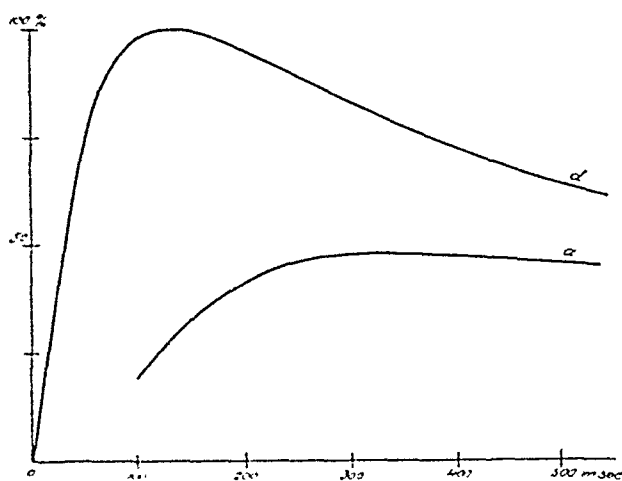
Discussion and Conclusions.

If the effect recorded from the optic nerve via directly coupled amplification only consisted of the summed nerve impulses, it should, generally speaking, form the frequency time curve of the nervous discharge. If such were the case, the inhibition of the impulses of the off-effect should cause the curve to fall towards the base line, and if all the impulses were stopped, the effect would fall entirely to the base line. There is, however, a *non-inhabitable remainder*, which if projected along the course of the off-effect usually appears as in fig. 8. This remaining potential rises slowly and falls gradually from a prolonged maximum 200—300 msec. after the beginning of the off-effect. After 500—600 msec. it falls as the contour of the off-effect.

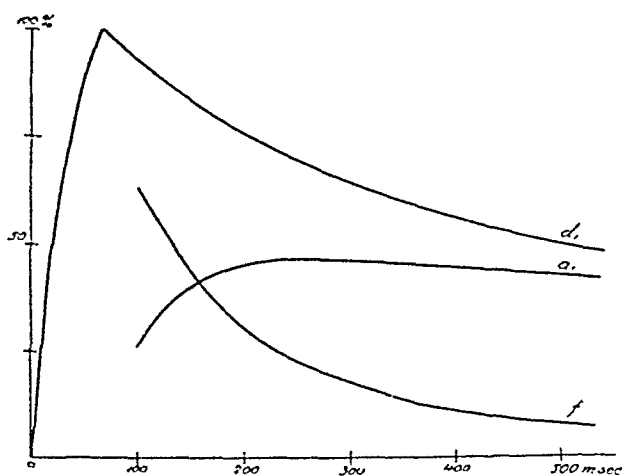
The non-inhabitable remainder rises at proximal recording to as much as 40 % of the maximum amplitude of the off-effect. This can scarcely be attributed to summed nerve impulses, it being probable that some of them remain uninfluenced by the inhibition of re-illumination (HARTLINE, 1938). The course and size of the non-inhabitable remaining potential as well as the "impulse free" contour of the curve during the inhibition period do not support a similar assumption (see figs. 5, 6, 9, and 10).

The diagram in fig. 13 B shows the data from 10 series of experiments with distal recording from the optic nerve. In five of these cases similar series of experiments have been carried out on the same preparation both from the proximal part of the nerve and the retina. The latter series are illustrated in fig. 13 A (retina) and fig. 13 C (nerve, proximal recording). In all diagrams the amplitude values are given in percentages of the maximum amplitude of the off-effect. A direct comparison can be made of the amplitude values in fig. 13 B and C, because in all these detailed

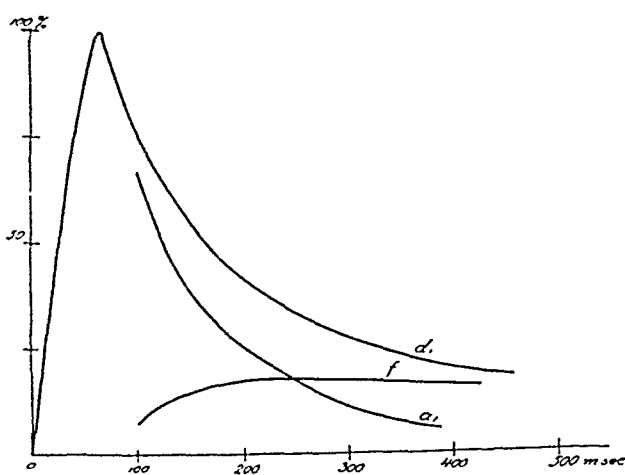
Fig. 13. Diagrams representing data obtained from the retina (A) and the optic nerve, "distal recording" (B), "proximal recording" (C) in ten series (see text) of experiments with re-illumination during the off-effect at varying intervals after cessation of light. \bar{d} and \bar{d}_1 average uninterrupted off-effect. \bar{a} and \bar{a}_1 average level of negativity occurring along the off-effect. f , difference between \bar{d}_1 and \bar{a}_1 . The amplitudes are given in percentages of the maximum amplitude of the uninterrupted off-effect. Time in msec. with zero time at the beginning of the off-effect.



A



B



C

experiments the off-effect gave the same amplitude both in distal and proximal recordings (about 0.4 mV). The maximal amplitude of the retinal off was about 0.6 mV.

The total off-effect shows quite a different picture in its general course in proximal recording (fig. 13 C) from the one we see when the active electrode is placed closer to the retina (fig. 13 B), although the initial maximal amplitude is of the same magnitude in both cases. The off-effect in proximal recording falls much more rapidly to the base line. The non-inhibitable remaining potential is considerably less, its amplitude scarcely reaching 50 % of the value in distal recording. There is no reason to suppose that a fraction of the summed nerve impulses would undergo such a considerable change with increased distance from the retina, all the more so as the initial maximal amplitude is identical in both recordings.

Thus, the high degree of amplitude diminution with increased distance from the retina furthermore strongly argues against the non-inhibitable part of the off-effect of the nerve being made up of summed impulses.

The remaining potential thus rather seems to be brought about by a slow potential change.

The diminution of amplitude of this slow potential with an increased distance from the retina seems to be the cause of the off-effect falling more rapidly to the base line in distal recording. The maximal amplitude of the remaining potential amounts to between 40 and 50 % of that of the off-effect, *i. e.* about 0.2 mV. Taken from a point 2—3 mm. more proximally, the remaining potential is then at most 15—20 % of the maximal amplitude of the off-effect or about 0.08 mV.

Within the period of the maximum off-effect when the impulse discharges are most frequent, the slow potential has risen very slightly. The total effect in this period consists mainly of summed nerve impulses and therefore the maximal amplitude of the off-effect in both recordings is of the same magnitude. As soon as the remaining potential rises and the impulse frequency diminishes, the d_1 and a_1 curves approach each other. Later on, 500—600 milliseconds after the beginning of the off-effect, the fall of the off-effect to the base line seems to be mainly determined by the course of the slow potential. Judging by these experiments, it seems as if the effect from the optic nerve, when illumination

ceases, is made up of a slow negative potential besides the summed nerve potentials.

It must be emphasized, however, that the potential illustrated (a_1), only forms a non-inhibitable remainder, after the elimination of the inhibitable fractions of the off-effect. The question now arises whether the part eliminated by the superimposed light contains any fraction beyond the inhibitable nerve impulses; in other words, does the superimposed light involve a potential change in the nerve resembling the notch of P III in the retina which is not to be attributed to any inhibition of the impulses?

Experiments so far carried out do not support such an assumption. In the majority of cases the superimposed light does not elicit any (fig. 10) or a very slight falling tendency (fig. 7) in the off-effect, when the inhibition occurs after the period of the maximum of the off-effect. In other cases a slight falling of the curve is visible (fig. 13), but this is only sufficiently great to attribute it primarily to the disappearance of the impulses in the summed effect. On the maximum of the off-effect where the impulses are most frequent and also the inhibitory effect most pronounced, the rapid fall of the curve must also be ascribed to the cessation of the impulses. A similar argument best agrees with GRANIT's and THERMAN's (1935) investigations of the impulse frequency in the case of superimposed light on the off-effect.

Should such a potential of an opposite sign accompany the impulse inhibition in the nerve, it might also be expected to diminish with the distance from the retina, as is the case with the non-inhibitable remaining potential.

In fig. 13 the curve f forms the difference between d_1 and a_1 . Both diagrams show fairly good agreement concerning the general course of the f -curve, when we take into consideration registration defects in relation to the complexity of the potentials obtained. Such a reliable agreement as this does not support the possibility of an opposite potential when electrodes are placed differently.

The f -curve thus represents approximately the general course of the real frequency time curve of the nerve impulses in the off-effect (cp. ADRIAN and MATTHEWS 1927 a; GRANIT and THERMAN, 1935).

On the basis of the results obtained, it will, however, be impossible to exclude a slow potential of the opposite sign appearing in the nerve with impulse inhibition. The experiments only point

to the fact that such a possible potential cannot be of such a magnitude — as is the case in the retina — that it makes itself visible in the method adopted and the degree of amplification used.

In earlier records of the action potentials from the optic nerve (*e. g.* WESTERLUND, 1912), a negative α -wave is also to be found. In consequence of this GRANIT has called attention *inter alia* to the difficulty existing in the analysis of the integratively registered nerve effect, the records easily becoming distorted owing to retinal phenomena, a circumstance also emphasized by GRANIT and THERMAN (1935).

Discussing the origin of the slow potentials identified in the off-effect of the nerve, we must exclude an effect from the retina spread simply electrically. Experiments show that no effect whatsoever is obtained from the nerve if it is pinched between the retina and the adjacent electrode (see figs. 9, 11 and 12), whereas the effect taken direct from the retina is fully pronounced. Thus the slow negative potential in the nerve will neither be caused by summed impulse potentials passing the electrode nor be due to simple electrical spread of the retinogram.

The fact that the amplitude of the potential diminishes with increased distance from the retina supports a retinal origin. For propagation along the optic nerve, it seems necessary, however, for the nerve to be intact. These circumstances seem likely to indicate that potential changes in the retina are spread by electrotonus to the extra retinal portion of the optic nerve.

Concerning the potential electrotonically spread to the roots of the spinal cord, BARRON and MATTHEWS (1938) found that the amplitude was diminished to half when moving the proximal electrode further away from the spinal cord. According to the investigations referred to, the slow potential in the optic nerve is reduced by more than 50 % when moving the active electrode 2—3 mm.

On the basis of their investigations with different recordings from the retina and the nerve, GRANIT and THERMAN (1938) emphasized the possibility of an electrotonic spread of slow potential changes in the retina. In addition to this, they found (personal communication) in different recordings from the retina and the nerve that when the latter was treated with cocaine, a slow potential remained after the disappearance of the impulses.

The fact that the slow potential found in the nerve should be of electrotonic character best agrees with the different results here obtained. Other interpretations must not, however, be excluded. There is the possibility that the slow nerve potential may possess qualities of a slow after potential. The picture obtained with both the electrodes on the living nerve (fig. 9) might indicate that the potential follows after the beginning of the impulse discharge. It is, however, quite possible that the slow potential in reality begins sooner, the initial increase being slow and followed by a steep rise after the first impulses in the nerve. The very earliest course of the slow potentials in these experiments cannot be determined with accuracy.

Moreover, when considering the results obtained, we must bear in mind the interesting fact that we are not dealing with a peripheral nerve but with a real central tract, *Fasciculus opticus* (KOPSCHE, 1935), with its typical histological qualities, which connects two nervous centres. Seen thus, the long potential discovered is of interest, whether it supports the electrotonic spread of slow potential changes in the retina or is to be regarded as a slow after potential. Arguments for impulse initiation by slow potentials (*e. g.* BARRON and MATTHEWS, 1938) and the knowledge of the relation between the after potentials and the level of excitability (*e. g.* ERLANGER and GASSER, 1937; GASSER, 1939) add to the interest of the different alternatives.

The question may be asked whether the slow potential changes appearing in the nerve can be compared with any of the specified components of the retinogram.

As has already been stated, the off-effect of the nerve at superimposed light does not show such pronounced momentary falling that it can be directly compared with the negative dip of P III in the retinogram. Nor does the normal on-effect (see figs. 7, 10 and 11) show any deflection under the base line corresponding to the pronounced *a*-wave of the retina (see fig. 3). Further, it is evident from preliminary experiments on a potassium treated eye that while the retina gives a well pronounced negative retinogram, no potential change whatsoever is obtained in distal recording from the nerve. These facts, again, contradict a simple electric spread of potential changes of the retina to the nerve.

Whether the nerve possesses a negative component functionally corresponding to the retinal P III is another question far more difficult to answer. As has already been pointed out, such a po-

tential may possibly exist, but if so, it must be of considerably less magnitude, as it has not been visible in the experiments.

It may be of some interest to compare the slow potential of the optic nerve with the non-inhibitable part of the retinal off-effect due to component P II. They have the same sign and generally speaking show the same course along the off-effect (cp. fig. 13 A and B). In addition to this they are bound to the same functional moments, for as slow negative potential changes, they appear in connection with stimulation.

With this comparison a similar slow potential change in the on-effect of the nerve would be expected, and that this seems to be the case will be seen in fig. 10 and fig. 11. The on-effect does not offer the same possibility for rational elimination of the nerve impulses, however, and therefore the possible slow potentials can not be selectively brought out. The records mentioned, however, go to prove a change in the on-effect with increased distance from the retina resembling that of the off-effect, which can hardly be considered a change in the summation effect of the impulses.

These investigations show the existence of a slow negative potential appearing in the optic nerve when the retina is stimulated by natural means. With the results hitherto obtained, it will be too early to determine whether the slow potential is a functional analogue to a fraction of the retinogram, *i. e.* the non-inhibitable off due to P II, or whether it is a slow potential electrotonically spread from the retina. The investigations so far carried out point rather to the latter fact.

The slow potential thus defined in the optic nerve is of functional interest on account of its appearing with the nerve impulses when the retina is naturally stimulated. The investigations carried out cannot be said to give evidence for the appearance of any slow potential change in the nerve with a sign opposite to that of the potential associated with excitation.

It may be pointed out that slow potentials of opposite signs can be recorded direct from the spinal cord, and are found to be associated with excitation and inhibition respectively (*e. g.* HUGHES and GASSER, 1934 a and b; see also ERLANGER and GASSER, 1937), while, (BARRON and MATTHEWS, 1938) when taken from the roots of the spinal cord, the potential changes only show negativity. In the ventral roots BARRON and MATTHEWS found that the development of the slow potential was checked by stimuli which cause inhibition of the impulse discharge. As an

analogy to this it may be pointed out how the slow potential in the off-effect of the optic nerve in its earliest stage is inhibited in its development by superimposed light (see figs. 10 and 12).

The slow negative potential in the optic nerve at off, the general course of which these investigations are intended to show, seems to possess in many respects qualities resembling the slow root potentials of the spinal cord. As its qualities are further analysed and its possible identity with certain fractions of the retinogram are further tested, several contributions will probably be made to the solution of the question concerning the localization and spread of the slow potential changes of the retina.

Summary.

1) In experiments on light-adapted frog's eye-nerve-preparations the retinogram and the action potentials of the optic nerve taken at different distances from the retina have been examined and have been compared at varying gaps of darkness in the adapting light.

2) The experiments illustrate, in agreement with earlier investigations (GRANIT and RIDDELL, 1934; GRANIT and THERMAN, 1935 and 1937; GRANIT and HELME, 1939), the relation between the negativity produced on the off-effect of the retinogram by the superimposed light and the impulse inhibition in the optic nerve.

3) The method adopted to inhibit certain fractions of the off-effect with superimposed light has been used to analyse more closely the integratively recorded off-effect of the optic nerve.

4) The experiments indicate that at the cessation of light a slow potential change takes place in the nerve besides the rapid nerve impulses.

5) The slow potential does not seem to originate from a simple electric spread of the retinogram or to be made up of summed impulse potentials passing the electrode.

6) The potential, which for its propagation requires the nerve to be intact, diminishes in amplitude by more than 50 % when the electrode placed nearest the retina is moved 2—3 mm. further away from the retina.

7) The slow potential change appears with negativity in the electrode situated nearest the retina.

8) The potential is checked in its development by stimuli (superimposed light) which cause impulse inhibition.

9) The experiments do not give positive expression for any slow potential change of opposite sign (positivity of the electrode situated nearest the retina) appearing in the nerve at impulse inhibition.

10) The possibility that the slow potential change in the nerve should be due to the electrotonic spread of slow potential changes in the retina is discussed. Such an assumption seems to fit in best with the experimental results.

11) The slow potential in the off-effect of the optic nerve is compared with the non-inhibitable part of the retinal off-effect due to P II. They show the same signs, have similar courses and stand in the same relation to functional moments.

PART II.

Observations on the Electrical Response to Light from the Optic Tectum in Frog.

Introduction.

In connection with the above mentioned experiments concerning some electrophysiological aspects of the excitatory processes in the retina and the optic nerve of the frog, attempts have been made to gather some information about the electrical response of the optic tectum of the frog when the retina is stimulated by light.

As has already been pointed out, the visual pathway forms an especially interesting structure from a neurophysiological point of view, the optic nerve as a true central tract linking the nervous centres of the retina together with higher ones. The activity of some ganglionic connections in the higher segments of the visual pathway have during recent years been studied in various quarters. The experiments have been carried out on rabbits and cats, and have mainly concentrated on the electrical response of the optic cortex.

The correspondence between certain structures of the cortex and the cortical representation of different senses have been demonstrated electrophysiologically (*e. g.* DAVIS, 1939).

The cortical electrical response to light stimuli (FISCHER, 1932; KORNMÜLLER, 1932) in the form of "evoked potentials" were proved to be confined to the area of the occipital cortex which corresponds to the structure of the *area striata* (*e. g.* KORNMÜLLER, 1937). This response from the optic cortex has been analysed in experiments on cats and rabbits, on which occasions both natural stimulation of the retina (*e. g.* BARTLEY, 1934, 1936 a and b; CLAES, 1939; FISCHER, 1932 and 1934; GERARD, MARSHALL and SAUL, 1936; KORNMÜLLER, 1932; WANG, 1934; and WANG and LU, 1936) and electrical shocks applied to the optic nerve were used (*e. g.* BARTLEY and BISHOP, 1933 a and b; BISHOP and O'LEARY, 1936 and 1938).

The analysis of BISHOP and his co-workers of the localized poten-

tials of the optic cortex seems to be the most complete yet available, and the results of other authors seem to agree with theirs in the main.

According to BISHOP *et al.* the response consists of three interfering series of potential waves. The first and most rapid of them consists of at least three rapid waves of a duration similar to axon spikes. The first of these is attributed to the axons of the afferent radiation, and the second to cortical neurones. The third, as opposed to the others, can be recorded below the cortex, and is considered by the authors to represent afferent fibres from the cortex to the superior colliculus. The second series of waves commencing at the beginning of the second spike potential consists of a primary slow surface-positive wave, followed by a slow surface-negative one, both having a duration of 5—10 msec. Finally a third series of still slower waves (duration 100 msec.) may follow.

(With reference to the widespread form of cortical response consisting in the abolition of the "general" potential brain rhythm see part III.)

The response of the optic tectum of the frog to light stimuli has not, however, been the object of investigations. A more detailed analysis is of special interest in consequence of the electrophysiological data obtained from the frog concerning the functions of the peripheral elements and their interconnections, the knowledge of which is essential for the study of the central connections of the visual pathway.

The frog offers, moreover, fairly primitive anatomical conditions, and the optic tecti are well separated and easily accessible for preparation. The optic tectum of the frog is generally composed of a central gray layer and a peripheral white one, some superficial portions of which consist of the optic afferent fibres which have undergone total decussation. The superficial optic bundle leads straight from the retina, while an axial bundle running more deeply passes the nuclei geniculati thalami on its way to the tectum (KAPPERS, HUBER, CROSBY, 1936).

Author's investigations.

The observations reported below should be regarded as a short appendix to the investigations concerning the slow potential changes in the optic nerve described in part I. They deal with potential changes obtained in different recordings from the optic nerve and the optic tectum in the frog. Although these investigations are of a preliminary nature, they have given results, however, which may be of interest.

Methods.

The apparatus was the same throughout, as described in part I (p. 20). In all experiments the direct-coupled amplifier was used. The same preparation was also used for the retina and the optic nerve. The nerve was kept in connection with the central parts, and the brain of the separated head was exposed. The retina was stimulated with "white light", and the effect was taken from the optic nerve and the contralateral optic tectum with the leads in different positions. The observations were not limited to any particular state of adaptation. In most cases the preparation was moderately light adapted. In the experiments published below, dealing with slow potential changes, the camera was running slowly.

Altogether some 300 records were taken of about 20 preparations.

Results.

Fig. 14 shows typical records from an experiment in which the potential changes in the optic nerve and the contralateral optic tectum was obtained when the retina was stimulated with light (on-effects) and with the electrodes in different positions. The diagram (retina — optic nerve — contralateral optic tectum) on the right of every record demonstrates how the electrodes were placed in each recording. Upward deflection indicates negativity of electrode I and positivity of electrode II.

Record 14 A shows the general configuration we know from the observations above (fig. 9), when both electrodes are placed on the living nerve. The record in fig. 14 A shows the effect more proximally than in the experiment illustrated in fig. 9. When the proximal electrode (II) is placed on the brain without touching the optic nerve or the tectum, and the distal electrode (I) is retained in the same position, a potential change of the shape illustrated in 14 B is obtained. The same result is obtained when the proximal electrode (II), instead of being placed directly on the brain, is brought into an indifferent (i) position elsewhere on the tissue of the head, as was in reality the case in fig. 14 B. In both instances a more or less pronounced initial upward deflection occurs, and this in its turn is followed by a potential change indicating slow positivity of the electrode (I) which remains on the nerve.

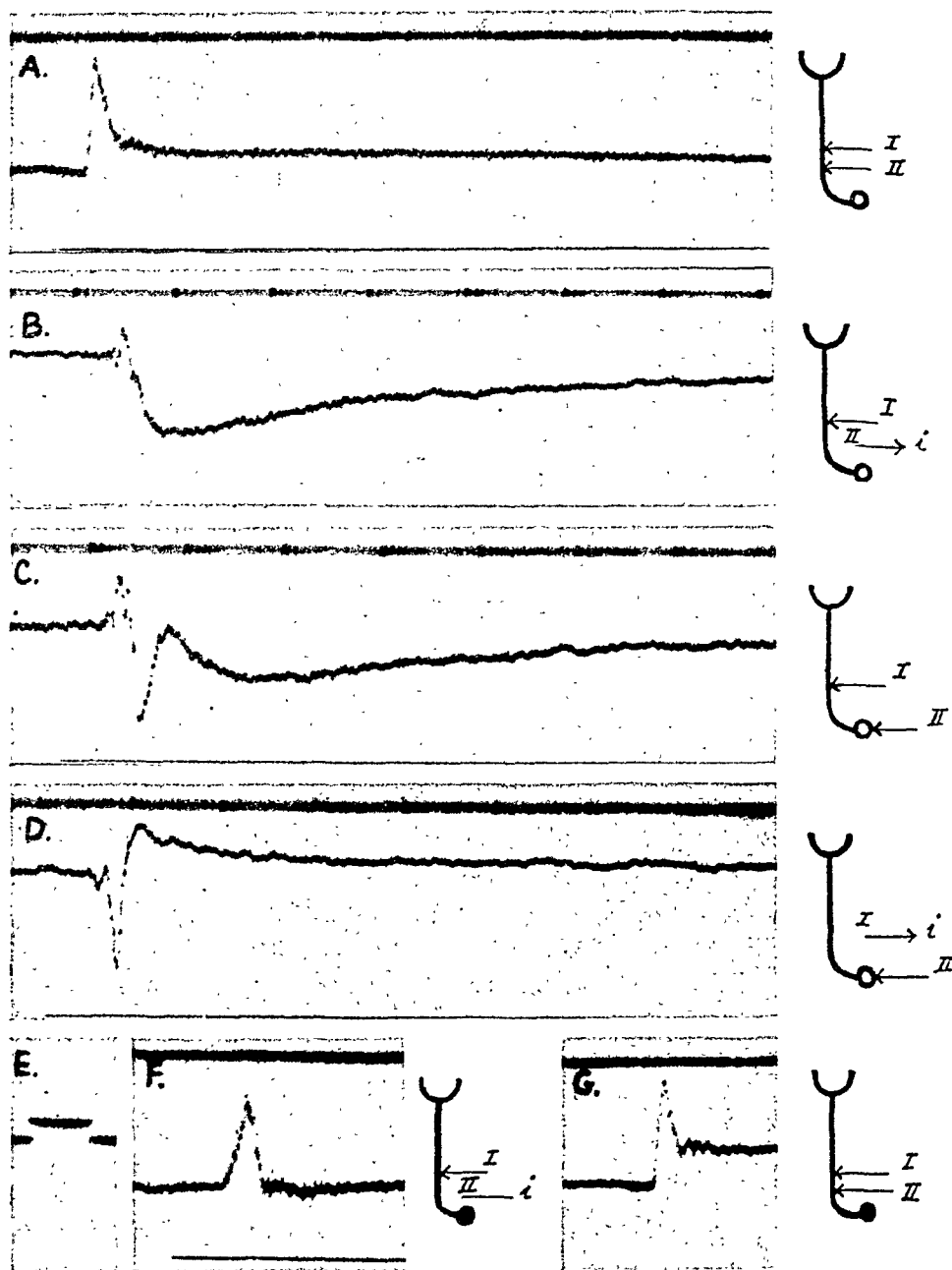


Fig. 14. Oscillograph record (direct-coupled amplifier) from optic nerve and optic tectum of frog with the electrodes (I and II) in different positions A—D and F—G as indicated in the diagrams (retina — optic nerve — optic tectum: intact ○ and killed or removed ●). E, calibration with 100 μ V. Time in $\frac{1}{2}$ sec. For further explanation, see text.

The picture in 14 C is obtained with electrode I still in the same position on the nerve, and electrode II placed on the upper surface of the contralateral tectum. As will be seen, the initial deflection is now more complicated, and is followed by an upward wave which seems to be written on the long downward deflection. With electrode II in the same position on the optic tectum and electrode I in an indifferent place, a monopolar recording from the tectum is obtained, the outline of which is to be found in 14 D, showing initial downward deflections followed by a slower wave upwards.

When the leads are replaced in the position as shown in fig. 14 B and any possible effect coming from the optic tectum is eliminated, the record shows a picture as illustrated in 14 F, whether the nerve is pinched in the vicinity of the decussation, or the whole of the optic tectum is killed (by means of prolonged potassium action) or removed from above. The configuration of record 14 F resembles, as was expected, the picture obtained in "proximal recording" in fig. 11.

Finally 14 G shows a record obtained after the electrodes had again been placed in their original positions, as seen in fig. 14 A.

All the records shown here demonstrate typical potential changes obtained at onset of light. Concerning the general shape of the off-effect, consistent results were obtained with the on-effect.

Discussion.

As is obvious from the records, the optic tectum of the frog gives no spontaneous rhythmic potentials of a sufficiently great magnitude to be visible with the amplification used. Even with intensified amplification, it is difficult to identify a spontaneous rhythm, which can definitely be distinguished from small accidental disturbances on the base line (cp. LIBET and GERARD, 1939).

When the retina is stimulated by light, a series of potential waves occurs in the optic tectum, the typical appearance of which is illustrated with monopolar recording in fig. 14 D. The potential response begins with a negative wave, with 2—3 crests or else is divided into 2—3 waves, on which small rapid potential oscillations seem to be superimposed. The amplitude of this initial negativity varies between 0.2 and 0.5 mV, and its duration is approximately 100 msec.

The negative potential complex is followed by a slow positive potential of varying duration with an amplitude which is generally somewhat lower than that of the negative potential.

No subsequent rhythmical potentials caused by light have been found.

The first visible upward deflection in record 14 B is probably to be regarded as the initial part of the potential of the summed nerve impulses (cp. fig. 14 A). This initial negativity in the electrode placed on the nerve is followed by a slow opposite deviation which will be referred to the electrode I being put on the nerve, it being of no consequence where electrode II is placed. It is also visible in the recording from the nerve (I) and the lobe (II, 14 C). The potential events from the nerve here merge with the potential changes from the electrode on the optic tectum. The result of this is a disintegration of the initial potential and a change of the following slow deflection, which can be explained by the slow positive potential of the tectum (14 D) being superimposed. It is an interesting fact that the slow positivity recorded from the nerve does not appear until after the initial nerve impulses (14 B), and also that it completely disappears when the optic tectum is killed or removed (cp. 14 B and 14 F).

In consequence of the different records discussed, it seems almost as though a slow potential change occurs in the nerve when this is connected to the intact tectum. In what manner the optic tectum contributes to its origin will not be discussed at the present stage. Both electric and physiological spread of the potential is possible.

Comparative experiments by placing the electrode I proximally and distally on the nerve show that the negative deflection attributed to the summed nerve impulses at distal recording has time to reach a more complete development before the positive deflection occurs, at the same time as the amplitude of the latter is lower. These experiments are of subordinate interest, however, as the potential events in the optic nerve discussed in Part I must be taken into consideration. It is peculiar, that the records 14 A and 14 G (before and after the elimination of the optic tectum) do not show any great difference, a circumstance that must be a warning against hasty conclusions concerning the interpretation of this slow potential change.

These preliminary and purely descriptive results at present do

not allow of any more detailed interpretation of the origin and nature of the frog's tectum response to light stimulus.

It is probable that one part of the initial negative deflections, obtained from the optic tectum (14 D), like the initial spike potentials in the records of BISHOP *et al.*, are to be attributed to the afferent fibres. Possibly some part of the negative complex is due to intertectal neurones, either as fibre potentials (cp. optic cortex, BISHOP and O'LEARY, 1938) or else as negative after potentials (cp. spinal cord, HUGHES and GASSER, 1934 a and b).

As an analogy, the following slow positive tectal wave might be compared with the positive intermediary potential led from the dorsum of the spinal cord, which GASSER *et al.* attributes to the internuncial neurones being a positive after potential (HUGHES and GASSER, 1934 a and b).

According to BARRON and MATTHEWS (1938), the intermediary positive potential led from the dorsal surface of the cord is the same as the slow negative potential of the dorsal roots, and arises, according to the authors, at the terminations of the dorsal root fibres themselves.

Whether there is any direct connection between the positive potential recorded from the surface of the tectum and the slow potential change in the optic nerve must remain an open question until the latter, which is indicated in fig. 14 B and C, has been more closely investigated.

Summary.

1. The electrical response of the frog's optic tectum, when the contralateral retina is stimulated with light, has been studied on moderately light adapted isolated head preparations.

2. The monopolar record of the tectal response, the general configuration of which is the same at "on" and "off", consists of an initial negative wave with two crests or is divided into two waves, followed by a more slow positive potential wave.

3. Recordings with the electrodes in different positions on the tectum and the optic nerve suggest a slow positive potential in the optic nerve following stimulation of the retina. This slow potential change seems to disappear when the optic tectum is killed or removed, or the nerve is pinched close to the decussation.

PART III.

Time Correlations in Man of Electrophysiological and Sensory Phenomena Following Light Stimuli.

Introduction.

Besides the "evoked potentials" which are localized to the optic cortex (see Part II), light stimulus produces a widespread form of electrical response from the cortex, which finds expression in an obvious change in the cortical rhythmic potentials.

The human brain potentials first registered by BERGER (1929) from intact skull show typical synchronization to certain definite frequency units. Among these dominates the one defined by BERGER as the *alpha rhythm* of about 10 waves per sec. Distinct limitation and predominance (GRASS and GIBBS, 1938), regularity (ROHRACHER, 1938) and constant rhythmicity (JASPER and ANDREWS, 1938; BERNHARD and SKOGLUND, 1939) are qualities of alpha rhythm which are visible in nearly all of the investigations made of human brain potentials. Alpha rhythm, which shows a certain periodicity (see fig. 15), is identified from almost any part of the skull (BERGER see 1938, ADRIAN and YAMAGIWA, 1935; LOOMIS *et al.* 1938; RUBIN, 1938). It shows the greatest amplitude (ADRIAN and MATTHEWS, 1934 b; ADRIAN and YAMAGIWA, 1935) and is most amply represented (RUBIN, 1938) when taken from the occipital region.

It has not been considered appropriate here to relate the whole extensive literature on human brain potentials under physiological and pathological conditions, some of which are of a purely descriptive nature and in this case of subordinate interest. The

literature has been exhaustively treated by several authors (*e. g.* by BERTRAND, DELAY and GUILLAIN, 1939; DAVIS, 1936 and 1939). It has likewise been considered irrelevant to go closely into the different opinions concerning the mechanism of autonomous rhythmic activity of central neurones represented partly by *e. g.* KUBIE (1930) and LORENTE DE NO (1934, 1935, 1938), and partly by *e. g.* GERARD (1936, 1937; DUBNER and GERARD, 1939; LIBET and GERARD, 1939).

At existing sequences of rhythmic waves, a light stimulus causes a disappearance of the regular rhythm. The effect, which in experiments on human beings is visible as a more or less complete abolition of alpha rhythm, has also been proved in the case of animals, when the experimental conditions have been such as to favour the rise of rhythmic brain potentials comparable with the alpha rhythm of human beings (ECTORS 1935 and 1936, rabbits). The phenomenon is easily proved from intact human skull and has been verified by most scientists in this field.

Diverging opinions as to the interpretation of the abolition phenomenon are to be attributed to various theories concerning the origin of the human brain potentials, which is at yet vague.

Experiments on animals have shown the dependence of the cortical activity upon the deeper cortical layers (*e. g.* DUSSER DE BARENNE and Mc CULLOCH, 1936 and 1938) and the sub-cortical connections (BREMER, 1937 and 1938 a and b), as well as that slow rhythmic waves can originate in the white matter of cortex (SJÖSTRAND, 1937).

BERGER, who was the first to discover the phenomenon, gave a generally formulated explanation founded on the opinion that every part of the cortex in activity is to be regarded as the source of alpha rhythm (1934, 1935, 1936). According to BERGER the activity in certain cortical areas caused by onset of light would produce widespread inhibition in adjacent cortical areas resulting in the suppression of the alpha waves.

In experiments on cortical response, JASPER (JASPER and ANDREWS, 1936; JASPER, 1936) tried to find expression for the changes in excitability comparable with observations from peripheral nerves (see *e. g.* ERLANGER and GASSER, 1937). With the general principles for peripheral nerves as a basis, JASPER endeavours to explain, *inter alia*, the cortical reaction to light. He maintains that the records of the human brain potentials show that the first period of the abolition (*alpha blocking*) is associated

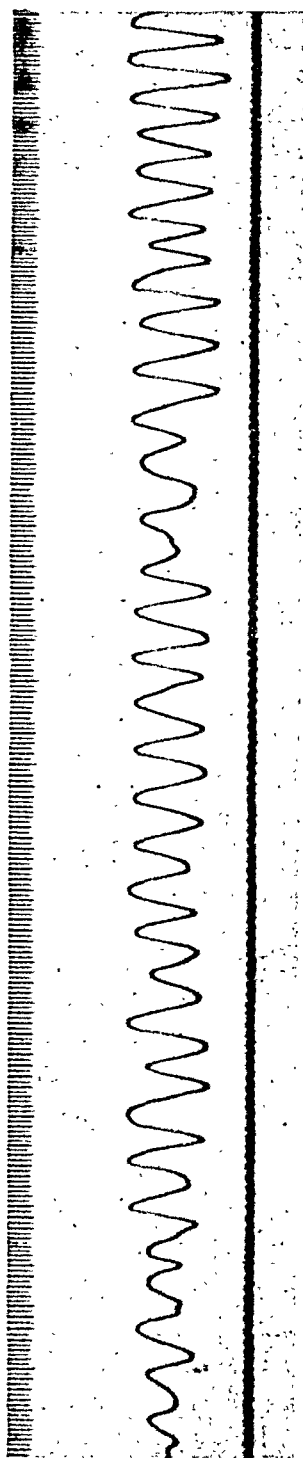


Fig. 15. Oscillograph record of the human occipital alpha rhythm. Time in $1/100$ sec.

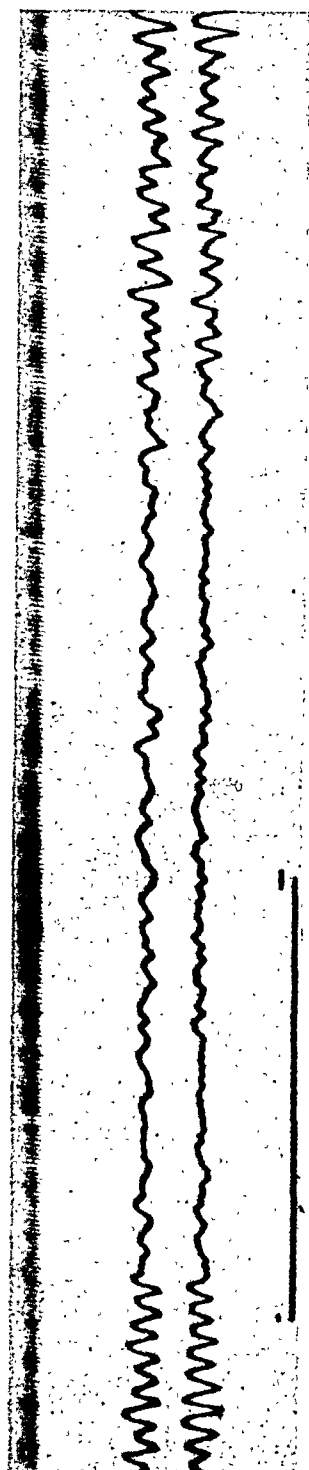


Fig. 16. Simultaneous oscillograph records of the alpha blocking to light taken from occiput (upper cathode ray) and vertex (lower cathode ray). Time in $1/50$ sec.

with slow positivity, which may be followed by slow negativity, and that a certain rhythmical activity may again be visible on the latter. The author compares these suggested slow potential changes with the negative and positive after-potentials, which, associated with supernormal and subnormal excitability, have been proved to exist in *c. g.* the peripheral nerves, the spinal cord (see ERLANGER and GASSER, 1937) and the superior cervical ganglion (ECCLES, 1935 a and b).

At the cessation of illumination the alpha waves gradually return after varying intervals of time (see *c. g.* ADRIAN *et al.* 1934 b and 1935; JASPER and CRUICKSHANK, 1937; CRUICKSHANK, 1937) and then show an increase of the frequency (CRUICKSHANK, 1937), which, according to JASPER (1936 b), may be as much as 40—50 %.

JASPER compares this phenomenon with the changes in the injury discharge of nerve fibres occurring during the response to an induction shock (GASSER, 1935), showing diminished activity during the positivity, followed by supernormal activity. Suggesting that the cortical potentials are central fibre potentials of slow time characteristics, JASPER explains these cortical reactions without resorting to other mechanisms than those which "can be demonstrated in the nonmedullated peripheral axon".

ADRIAN and MATTHEWS (1934 b) as well as ADRIAN and YAMAGIWA (1935) carried out extensive series of experiments to prove BERGER's observations concerning the characteristics of the human brain potentials. In experiments with the electrodes in various places, they found that the amplitude of the alpha waves is greatest over the occipital region, and decreases the further away the electrodes get. Their experiments with intermittent light show how the potential waves in certain limits can be made to follow the frequency of the flickering light (*cp.* later observations of LOOMIS *et al.*, 1936; JASPER 1936; DURUP and FESSARD, 1935; GOLDMAN, SEGAL and SEGALIS, 1938). The rhythmic potentials then show a distribution over the head with the frequency of the flicker instead of the usual 10 pr. sec. rhythm. The alpha blocking was also seen to be most satisfactorily pronounced over the occipital region. The authors point out that the most important factor for the appearance of the alpha rhythm is the absence of pattern vision, and as BERGER points out, rest in darkness gives the best conditions for the registration of the alpha frequency.

According to ADRIAN *et al.*, the experiments point to the fact that alpha rhythm arises in consequence of co-operating spontaneous discharges from a large group of cortical units with occipital localization. When these neurones are free from disturbing stimuli, they beat in unison at their natural period. The light stimulus produces a non-uniform excitation causing desynchronization. According to ADRIAN *et al.*, the alpha blocking thus gives expression for asynchronized activity in certain cortical areas mainly concerned with vision.

ADRIAN's investigations (1937 a) of the synchronized reactions in the optic ganglion of the water-beetle (*Dytiscus marginalis*) give an interesting analogue to the alpha blocking reaction in the cerebral cortex. Fresh preparations of the *Dytiscus* optic ganglion give no spontaneous rhythm in darkness, while stimulation of the receptors with strong light causes potential oscillations in the ganglion ("bright rhythm"). The regular potential changes commence with a frequency of 20—40 per sec. slowly to approach 15—25 per sec. at continued illumination. If the preparation is left for some hours, a strong spontaneous rhythm in darkness of 7—10 waves per sec. ("dark rhythm") gradually starts. In this state bright illumination gives blocking of the dark rhythm. In the former case weaker intensities of stimulus give an irregular discharge, while in the latter they produce an incomplete blocking of the dark rhythm. The potential waves of the ganglion, which are in both cases associated with corresponding groups of impulse discharge in the post-ganglionic nerve must, according to ADRIAN, be interpreted as synchronized activity in a large number of units.

The definite potential rhythms represent states favouring the synchronization of the activity of the separate elements. As the experiments show that the neurones respond over a wide frequency, ADRIAN maintains that a fixed frequency response in the neurones contributing to the waves cannot be assumed. The main assumption for synchronization seems to be that the generating units possess the same degree of excitation, so that they beat with the same frequency. The greatest probability for this is to be found at the extreme ends of the visual scale, *i. e.* at bright illumination and complete darkness. Bright stimulation brings about discharges of maximal frequency, while in darkness the neurones beat at a minimal rate ("resting discharge"). Both states favour the synchronization of the activity. The fact that

the dark rhythm only occurs in the ganglion after it has suffered injury, — on account of its being left in a reclining position — would, according to ADRIAN, be due to the fact that the slight injury produces a breakdown in the normal insulation. For the sake of comparison, ADRIAN points out similar effects of injury in the phrenic nerve (ADRIAN, 1930) and in the lateral line nerve (HOAGLAND, 1933). Thus according to ADRIAN, the blocking of the rhythmic potentials seems to arise when the stimulated neurones respond to different frequencies causing a desynchronized activity.

The synchronized effect in the optic nerve of the eel (ADRIAN and MATTHEWS, 1928) and the frog (GRANIT and THERMAN, 1935) must be remembered in this connection. The synchronization of the fibre impulses are best seen in the off-effect after strong stimuli (GRANIT and THERMAN, 1935; see also figs. 5, 6, 7, 9 and 10) but also in the on-effect in connection with stimulation of a large part of the retina.

The tendency of *Dytiscus* preparations to give definite potential rhythms, which in certain circumstances can be made to disappear without noticeable frequency changes, offer an amazing resemblance to the alpha blocking in the cerebral cortex.

The comparison between the potential changes in the *Dytiscus* optic ganglion and the human brain potentials (ADRIAN, 1937 a) implies, according to ADRIAN, that a fixed frequency of the cortical neurones need not necessarily be pre-supposed for the genesis of the alpha waves (cp. ADRIAN and MATTHEWS, 1934 a). When the cortical neurones are free from external stimuli, they beat at their minimum rate, causing the synchronization of the constant alpha rhythm. The breakdown of this takes place in consequence of a non-uniform excitation of the cortical units, which is primarily caused by light stimuli.

The above formulated opinion of ADRIAN *et al.* concerning the alpha blocking, formed after experiments on human beings, seems to find a certain support in the results from comparable experiments on simpler objects. Their explanation is also supported by observations made in various quarters concerning the rhythmic discharges in the nervous system (see *e. g.* ADRIAN, 1932 a, 1937 b).

The analysis of the alpha blocking phenomenon, however, is obscure owing to several complicating factors.

On the basis of their experiments with simultaneous recording from several points on the head, ADRIAN and YAMAGIWA (1935)

favour two occipitally localized main foci for the alpha rhythm, which they attributed to the two occipital lobes. The results, too, seem to indicate that these foci change positions within certain boundaries. In this connection they point out that the alpha rhythm from different parts of the head shows obvious differences in phase between the different regions. It has subsequently been asserted from different quarters that these phase differences are of such a nature that there is reason to suppose that further foci for the origin of the alpha frequency are localized to other regions as well (JASPER and ANDREWS 1938; LINDSLEY, 1938 b; JANZEN and KORNMÜLLER, 1939). The alpha rhythm with its characteristic qualities is, however, always identified as the dominating rhythm in the different regions (see also DAVIS and DAVIS, 1936; LOOMIS *et al.*, 1938; RUBIN, 1938).

The blocking reaction, as has been proved from several quarters, is not peculiar to light stimuli, but is also caused by other sensory stimuli (*e. g.* tactile, acoustic), which has been proved both in experiments on animals (*e. g.* ECTORS, 1935; REMPEL and GIBBS, 1936) as well as on human beings (ADRIAN and MATTHEWS, 1934 b; DURUP and FESSARD, 1935; JASPER, 1936 b; JASPER and CRUICKSHANK, 1937; ROHRACHER, 1937; LOOMIS *et al.* 1938; TRAVIS and BARBER, 1938).

As was pointed out by ADRIAN and MATTHEWS (1934 b), the alpha blocking seems, however, to be most intimately associated with light stimuli, which produce the most constant and best pronounced effects. It seems to be characteristic that just as the typical alpha frequency is identified from different regions, the blocking of the alpha rhythm after sensory stimuli seems to take place simultaneously over the whole cortex. LOOMIS *et al.* (1938), who by stating the time in 1/100 sec. proved that such seems to be the case, sum up their observations with the following words: "With respect to disturbance potentials appearing on the surface of the skull, the cortex acts as a whole" (see fig. 15).

While the blocking of the alpha rhythm after light stimuli can be recorded with the greatest facility from intact human skull, it seems doubtful whether a local effect originating from the optic cortex (cp. part II) can really be identified under these experimental conditions.

In a few cases (JASPER and CRUICKSHANK, 1937; CRUICKSHANK, 1937; JASPER and ANDREWS, 1938), a similar local on-effect has

been suggested, which seems to offer resemblances to the on-effect demonstrated on animals (see Part II). According to JASPER, this on-effect is best seen if the light stimulus is given in a period when the alpha rhythm is lacking, for example, in the blocked period after a previous light stimulus. The amplitude and latency of the local on-effect, according to CRUICKSHANK, would be a function of the stimulus intensity, and she gives the value of the latter as 0.06 seconds for higher light intensities.

Some authors have pointed out, though without any actual experimental basis, that the flickering potentials proved by ADRIAN and MATTHEWS might possibly be regarded as evoked potentials (LOOMIS *et al.* 1938).

The prolongation of the latency for the blocking of the alpha rhythm (blocking time according to JASPER) with decreasing light intensities was first observed by DURUP and FESSARD (1935, 1936). They show in a series of experiments on a person with marked blocking of the pronounced alpha rhythm a continual prolongation of the blocking time with decreasing intensities. CRUICKSHANK's (1937) experiments on the blocking time as a function of the stimulus intensity show in principle the same result. By plotting blocking time against log brightness in some cases, however, she finds a diphasic course in the curve thus obtained. The diphasic curve is compared with the dark adaptation curve (HAIG and WALD) presented by HECHT (1934), which would imply that the two phases would reflect rod- and cone-functions respectively.

Author's Investigations.

The investigations to be recorded below thus centre around some time characteristics of the blocking reaction of the human occipital cortex, showing the time relation between the retinal and cortical electrical response and the relationship between these peripheral and central electrical responses to light and to the perception of light.

General Apparatus.

For registration a Loewe cathode ray tube (cp. page 20) with two beams was used.

Amplifiers: For recording human brain potentials the oscillograph was used in conjunction with two different amplifiers. The 4-stage push-pull coupled amplifier (page 20) with a resistance-capacity-coupling at the input (calibration see fig. 17 A) and a 4-stage condenser coupled amplifier with 2 push-pull initial stages were used alternatively. The amplifiers were generally used with shunted inter-stage-coupling capacities in order to eliminate frequencies of above 20 p/s (calibration fig. 17 B). In order to see that the different condenser-couplings did not cause any errors when determining the latency for the alpha blocking, the effect thus obtained was controlled by comparing with that obtained by direct coupled amplification.

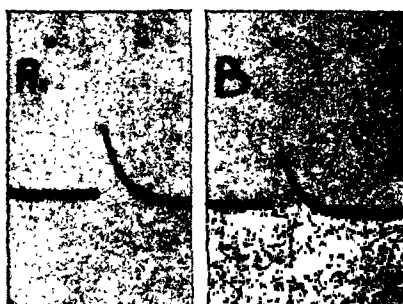


Fig. 17. Calibrations with 30 μ V of direct-coupled amplifier when using resistance-capacity coupling at the input (A) and shunted inter-stage-coupling capacities (B). Time in 1/5 sec.

For recording the human retinogram the 4-stage push-pull coupled amplifier was used direct-coupled (calibration fig. 2 A and B). At simultaneous registration of the retinogram and brain potentials this was coupled to the one beam of the oscillograph, while the condenser coupled amplifier was connected to the other beam used for the brain potentials.

In the experiments in which muscle action potentials from biceps were recorded, the condenser coupled amplifier was used.

Electrodes: Ag—AgCl electrodes were used in all instances. Monopolar recording of the brain potentials was used, the active electrode being placed over the occipital region in the midline and the indifferent electrode on the right processus mastoideus (ADRIAN and MATTHEWS, 1934 b; RUBIN, 1938). The electrodes used were of the construction indicated by BERNHARD and SKOGLUND (1939), comfortably fixed by rubber bands.

The same electrodes were used for recording the muscle action potentials. They were then placed over the biceps at a distance of 5 cm. from each other (cp. ALTENBURGER, 1937). For the recording of the retinogram, the one electrode consisting of a chlorinated silver thread in communication with a wick soaked in physiological NaCl solution was placed in the anaesthetized (novocaine) conjunctival sac (cp. COOPER, CREED and GRANIT, 1933). The other electrode

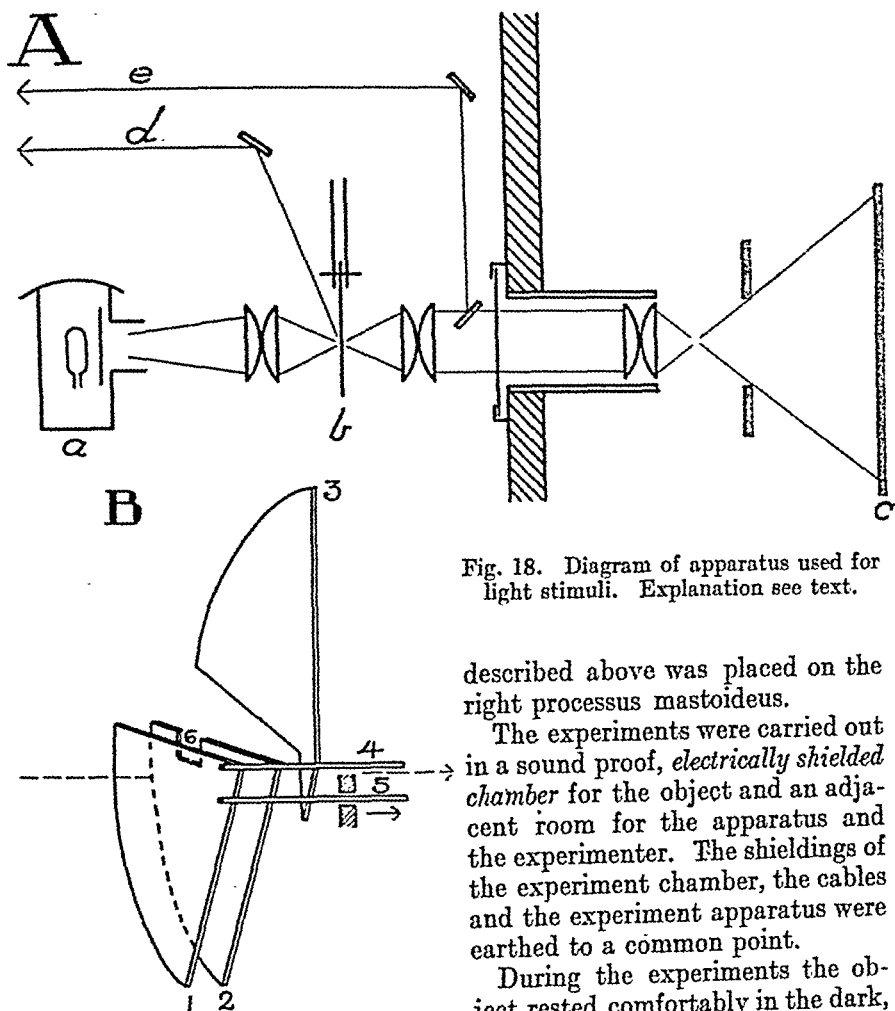


Fig. 18. Diagram of apparatus used for light stimuli. Explanation see text.

described above was placed on the right processus mastoideus.

The experiments were carried out in a sound proof, *electrically shielded chamber* for the object and an adjacent room for the apparatus and the experimenter. The shieldings of the experiment chamber, the cables and the experiment apparatus were earthed to a common point.

During the experiments the object rested comfortably in the dark, undisturbed by accidental stimuli, while the recording took place in the adjacent room from which the light stimuli were also distributed.

Under these circumstances special arrangements had to be made for the distribution of the light stimuli, which had also to function both noiselessly and at the same time to fulfil the requirements essential for the experiments described in page 80.

The apparatus for the distribution of the light stimuli is illustrated schematically in fig. 18 A and B. "White light" was obtained from a 200 watt Osram projection lamp (a) in a shaded lamp holder with a circular hole for the outgoing beam of light. Passing a system of lenses, the beam was conducted through a tube into the experiment chamber, then spread and projected on a reflecting white screen (c) before the object. The light was concentrated by a condensor lens before the passage into the chamber. In the focus (b) there was a special arrangement placed for the onset and the interruption of the light. This apparatus is illustrated schematically in fig. 18 B. Three sector-formed screens

(1, 2 and 3) made to turn round an axis (4) are held in the position given in the diagram by means of a block arrangement (5). When this is pushed one step in the direction of the arrow by a distance controller operated by hand, the screen 1 falls. The light then passes through a filter (6) in the second screen. When the block is pushed another step, screen 2 falls and then the light is let in with its full intensity. A last push of the block brings about the interruption of the light when the third screen falls to take up the position given in the diagram for 1 and 2. The screens are checked in their fall to the desired position by special brakes. The apparatus makes it possible to superimpose stronger light on a former weaker light stimulus in a simple manner. The intensity of the two stimuli can be varied by putting in filters with different densities, both in screen 2 and in a holder placed just behind screen 3. For the distribution of only one light stimulus, screen 2 is dropped to a position where it does not function, and light is put on and off by means of screens 1 and 3, as described above. A little mirror fixed to the edge of the first screen throws a small beam when the screen falls, which via other mirrors (see fig. 18 A, e) produces marks on the bromide paper to indicate the onset of the first light stimulus on the records. In the same manner a little mirror is inserted behind the apparatus to divert a small beam of light in order to mark the stronger superimposed light stimulus.

Written neutral tint filters were used for the variation of the intensity of the stimuli. In the experiments published below the stimulating disc was of 40 cm. in diameter at a distance of 60 cm. from the eye of the object, making 37° of the visual angle and without any filter of about 10 ml. brightness.

This light intensity will be referred to in the text as 1, other intensities as fractions of 1.

Time markings were obtained from a 50 (c./sec.) generator coupled either to the one beam or to a neon lamp with a fine slit.

The camera was driven by a motor giving no disturbances.

Relation between Intensity of Visual Stimulus and Blocking Time of Human Occipital Alpha Rhythm Compared with Intensity-Latency Relation of Action Potentials in the Optic Nerve of the Frog.

Blocking Time of the Human Occipital Alpha Rhythm.

Technique.

In each particular case considerable variations occur concerning the predominance of the alpha rhythm in the frequency spectrum obtained. By means of automatic FOURIER Transform, GRASS and GIBBS (1938) clearly demonstrated the predominance

of the alpha rhythm, even in cases where it was difficult to identify on direct inspection.

In the following only such cases have been selected which show clearly pronounced alpha rhythm, so that the blocking caused by the light stimulus is distinctly marked.

In order to obtain a constant sensitivity in the retina, suitable for the intensity scale tested, the subject was dark adapted for about 30 mins. before the experiment took place. After this time the decrease of the intensity threshold in the retina approaches asymptotically final value (KOHLRAUSCH, 1931).

The subject lay comfortably in the dark. Attention was paid to avoid drowsiness and sleep, conditions which change the brain potentials and the cortical reactions (see BLAKE and GERARD, 1937; DAVIS *et al.* 1938, 1939; LOOMIS *et al.* 1936, 1937, 1938). The experiments were carried out on one eye. At each experiment several intensities were tested and each light stimulus lasted for about 1 sec. Between each of these there was an interval of at least one minute, partly to give any possible after-image time to disappear (JASPER and CRUICKSHANK, 1937; TRAVIS and HALL, 1938), and partly to give the alpha rhythm time to return to the normal frequency value after the rise that usually appears after the blocked period (JASPER, 1936 and CRUICKSHANK, 1937).

In order to obtain uniformity and a correct judgement of the experiments, attempts have been made to put in the light stimuli in periods of well pronounced alpha rhythm.

Results.

Experiments carried out under these conditions show a striking blocking of the alpha rhythm after the light stimulus (see figs. 16 and 19). The blocking usually takes place with a sudden interruption in the regular rhythm (see fig. 19 A—D). The last alpha wave sometimes shows somewhat diminished amplitude before the blocking, a condition which is generally more often visible after low light intensities (fig. 19 E). The phenomenon is of varying nature, and a possible temporary diminution of the amplitude quite independent of the light stimulus cannot be excluded (cp. fig. 15). This fact sometimes makes it difficult to form an exact judgement of the blocking time.

Fig. 19 shows typical records from a series of experiments in which six different light intensities have been tested, and illustrates the prolongation of the blocking time with decreasing intensity.

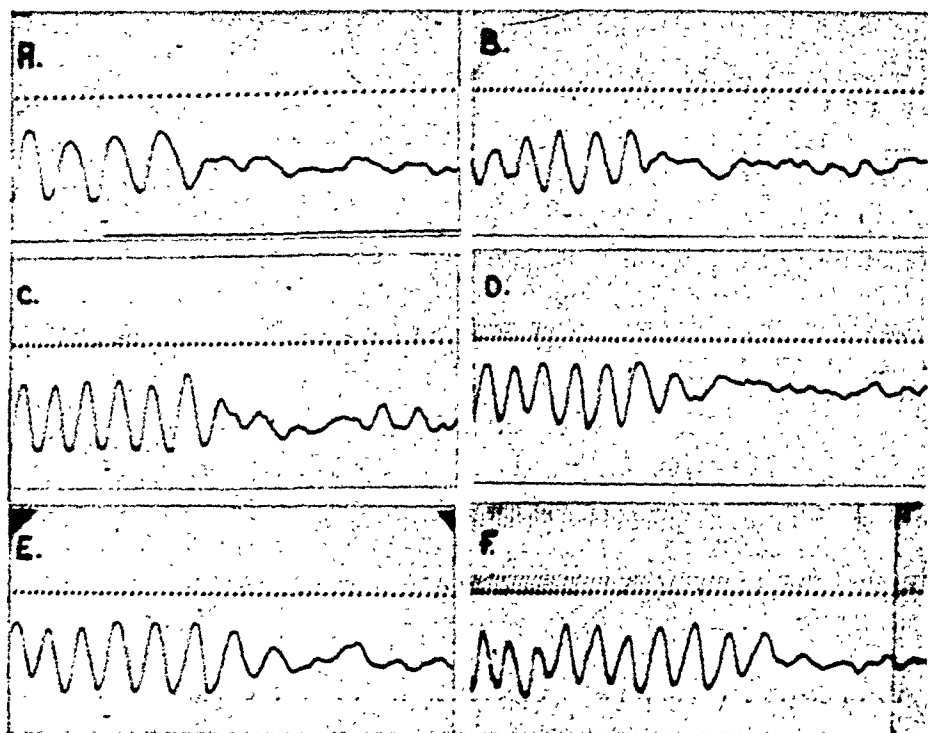


Fig. 19. Oscillograph records showing the blocking of the occipital alpha rhythm to light at intensities 1 (A), 1/10 (B), 1/100 (C), 1/1,000 (D), 1/10,000 (E) and 1/100,000 (F). Time in 1/50 sec.

Table 1 clearly shows the good agreement in blocking time at different intensities when the experiments are carried out under the conditions stated above. The values are given according to the reading in 1/100 seconds. It should be pointed out that the variations, besides being caused by the possible biological variations, are also largely brought about by the variations in the evaluation of the readings, as stated above. As is seen, the deviation from the average remains within reasonable limits.

In fig. 20 the average values for the blocking time at different light intensities obtained in experiments on five different human subjects are plotted against log brightness. The different persons are represented in the figure by the five different types of circles marking the individual average values.

As is evident from the diagram, the values for the blocking time at the different intensities for the different persons are of the same magnitude. The blocking time is seen to increase continually with decreasing intensities of stimuli. The curve graphic-

Table 1.

Blocking time of occipital alpha rhythm in 1/100 sec. at intensity:			
	1	1/1000	1/10000
	15	26	30
	15	24	38
	18	28	40
	17	30	41
	15	28	38
	18	38	33
	17	28	32
	13	25	40
	20	30	31
	17	30	37
	16	30	40
	17	38	40
	20	28	38
	14	36	34
	17	28	32
	14	30	34
Averages:	16.4 ± 0.5	29.8 ± 1.0	36.1 ± 0.9

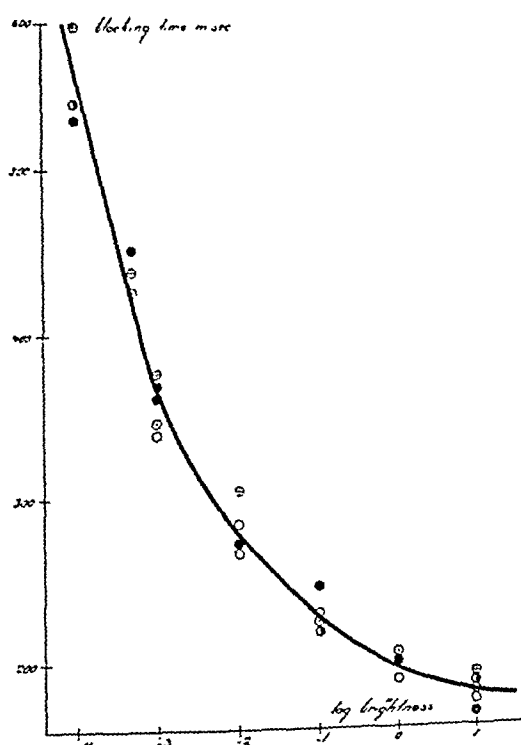


Fig. 20. Plot of blocking time as a function of log brightness. The values for the blocking time at different light intensities are obtained from five different subjects represented by the five different types of circles marking the individual average values. Time in msec.

ally obtained shows the general relation between the blocking time and the log brightness within the range tested of the visual scale, which lies between the value for the visual threshold and the intensities at which the blocking time asymptotically approaches a minimum value.

Latency of Action Potentials in the Optic Nerve of Frog.

Technique.

The action potentials were recorded by means of the condenser coupled amplifier described by GRANIT and SVAETICHIN (1939) with short time constant and balanced input stage. In addition to this the registration apparatus described on page 20 was used.

Both "white light" and monochromatic light was used. The spectral light was obtained from a TUTTON monochromator described by GRANIT and MUNSTERHJELM (1937), which was used according to GRANIT's and SVAETICHIN's (1939) instructions. The same technical arrangements with regard to "white light", preparation etc. were made as previously described on page 20.

The experiments were made on preparations from dark-adapted frogs, which were kept in the dark and in room temperature for at least 12 hrs. before the experiment was commenced, and the preparation was carried out in red light (see THERMAN, 1938).

Exposures were made at intervals of 2 seconds.

Results.

The latency for the impulses in the optic nerve was studied as a function of intensity by using "white light" and monochromatic light at 0.470μ , 0.540μ and 0.570μ .

Fig. 21 shows the latency for the optic impulses at six different light intensities of monochromatic light at 0.540μ . The diagram in fig. 22 shows the latency as a function of log brightness at 0.540μ . The curve shows an even monophasic course within the range tested, at the highest intensities of which the latency asymptotically approaches a minimum value.

The same general course in the intensity-latency curve was obtained by using "white light" and spectral light at 0.470μ and

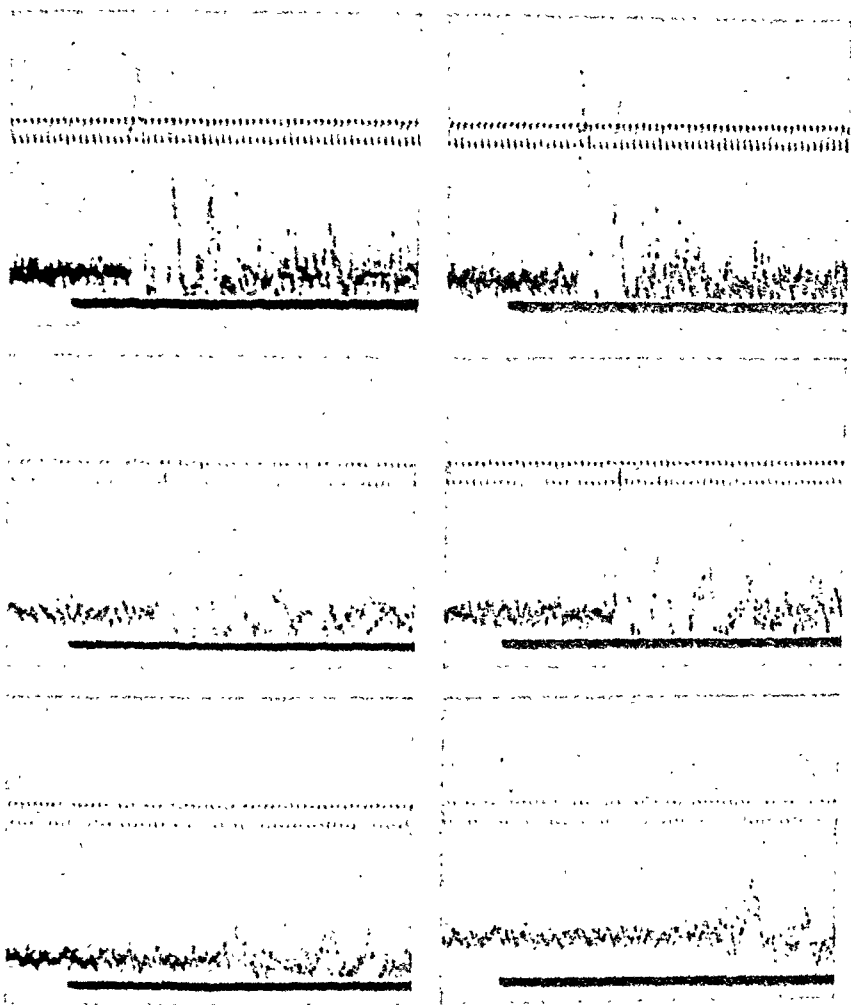


Fig. 21. Oscillograph records showing the action potentials in the optic nerve of dark adapted Hungarian frog at light intensities 1 (A), 1/10 (B), 1/100 (C), 1/1,000 (D), 1/10,000 (E), and 1/100,000 (F). Wave length 0.540μ . Time in 1/100 sec.

0.570μ . In each case the course illustrated in fig. 22 was obtained, here being no indication of any break in the curve in any single case.

Discussion.

Earlier investigations of the latency of the action potentials in the optic nerve (ADRIAN and MATTHEWS, 1927 a and b, 1928, conger eel) have already been mentioned (see page 17) as well as the discussion of the relation between the latency of the optic nerve potentials and that of the retinogram (ADRIAN and MAT-

THEWS, 1927 a and b, 1928; GRANIT, 1933; GRANIT and HELME, 1939; see page 25). With the background of the investigations mentioned, the course of the curve illustrated in fig. 20 seems to show that the prolongation of the blocking time must be primarily due to a prolongation of the latency time of the peripheral processes. The diagrams in figs. 20 and 21 illustrate curves obtained at comparable intensities from a physiological point of view, where

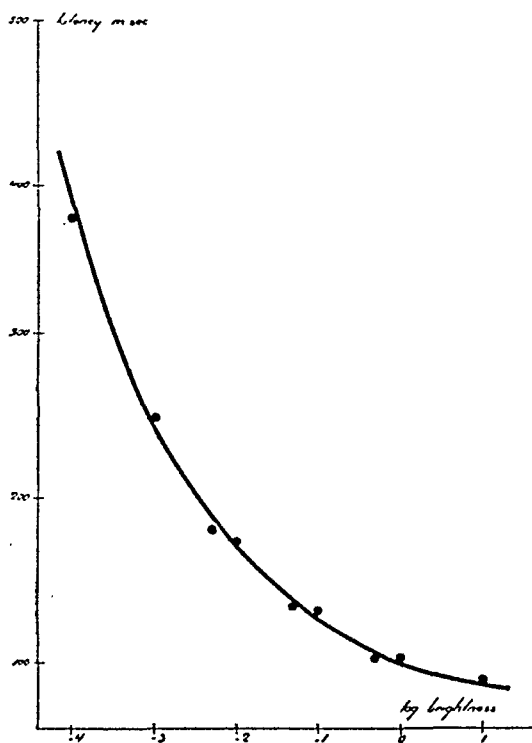


Fig. 22. Plot of latency of impulses in the optic nerve of dark adapted Hungarian frog as a function of log brightness. Wave length 0.540μ . Time in msec.

the latency curves asymptotically approach the minima at the highest intensities. The curves show general agreement as regards their general course.

Although the results have been obtained from various subjects and thus only offer relative comparison, they will nevertheless give distinct support for the assumption that the prolongation of the latency in the response recorded from the centre must be to a great extent caused by the prolongation of the latency time of the peripheral response.

CRUICKSHANK (1937) pointed out the fact that the curve for

the blocking time would reflect the peripheral latency curve. According to her experiments, the curve for the blocking time in certain cases may show a diphasic course, and she also points out that the two phases may be attributed to rod and cone functions respectively.

If this is really the case, the latency curve for the optic nerve impulses in the frog, whose eye like that of the human being is a typical mixed rod and cone eye, should show a diphasic course. No such results were obtained, however, in the experiments described above. The relation between intensity and latency have been investigated by using both "white" and spectral light. In the latter case monochromatic light was used in the cone spectrum, in the rod spectrum and at the wave lengths, at which both elements give high response, judging by the rod and cone curves for size of *b*-wave against wave-length (GRANIT and MUNSTERHJELM, 1937; GRANIT and WREDE, 1937). If the above mentioned deviations in the course of the latency curve were to be attributed to different qualities in the rod and cone elements, they ought most probably to appear at 0.540μ ; this curve, here as well as in the other cases, however, shows a regular monophasic course.

HARTLINE's (1938) previously mentioned investigations, as well as GRANIT's and SVAETICHIN's recently (1939) published observations on the distribution of sensitivity in the spectrum for different elements in the retina, give interesting criteria for the functional specificity of the optic nerve fibres. It is as yet an open question, however, whether the different receptor functions are associated with fibres of different diameters. If such should be the case, the results obtained are nevertheless hardly unexpected, considering the continuous variation in diameter of the optic nerve fibres of the frog (BURSTEIN and LÖNNBERG, 1937).

The measurements made by HOLMGREN (personal communication) of the fibre size in the human optic nerve show a distribution curve generally resembling that of the frog, with the exception of an insignificant second hump indicating a small subdivision of larger fibres. Having as yet no knowledge of the relation between the functional specificity of the optic fibres and their size, it is impossible to say whether on account of this circumstance there is any reason to expect a course in the blocking time curve which deviates from that of the latency of the optic impulses in the frog.

These investigations do not in any way indicate a diphasicity in the curve for the blocking time. The results point at a regular monophasic course of this curve.

Investigations of the Retinal Action Potential of Human Eye with Special Reference to the Relation between Latency of the Retinogram and Blocking Time.

The retinal action potential of the human eye was first registered by DEWAR and M'KENDRICK (1873, 1874; DEWAR, 1877) and later by HARTLINE (1925), KAHN and LÖWENSTEIN (1924), SACHS (1929), and KOHLRAUSCH (see 1931).

COOPER, CREED and GRANIT (1933) subjected the human retinogram to a closer analysis in connection with GRANIT's investigations (1933) of the action potentials in the cat's eye, and their records give the general characteristic of the human ERG at continuous and intermittent illumination.

The retinogram of the human dark adapted eye lacks the negative *a*-wave, shows a prolonged *b*-wave with low amplitude (maximum 0.2 mV against 0.5 mV in the cat at similar recording), an insignificant *c*-wave and lacks a definite off-effect. The latency is long, and according to GRANIT *et al.* goes up to 60 msec. at 10 ml. brightness. GRANIT *et al.* found in agreement with SACHS (1929) that flickering light gave a wavy response.

Records published by GRÖPPEL, HAAS and KOHLRAUSCH (1938) show a retinogram of a similar shape. The authors point out the existence of certain periodical potential changes, which they attribute to the periodical after-images.

Technique.

The subject was dark adapted previous to the experiment and the chamber was almost dark when the electrodes were fixed.

The light stimuli were given at intervals of approximately one minute. The intermittent lightstimuli were obtained by a rotating flickering screen driven by a motor giving no disturbances.

Altogether about 15 experiments were carried out on different subjects and some 400 records were taken.

Results.

The record in fig. 23 A shows the characteristics of the human ERG., as pointed out by GRANIT *et al.* This retinogram which is

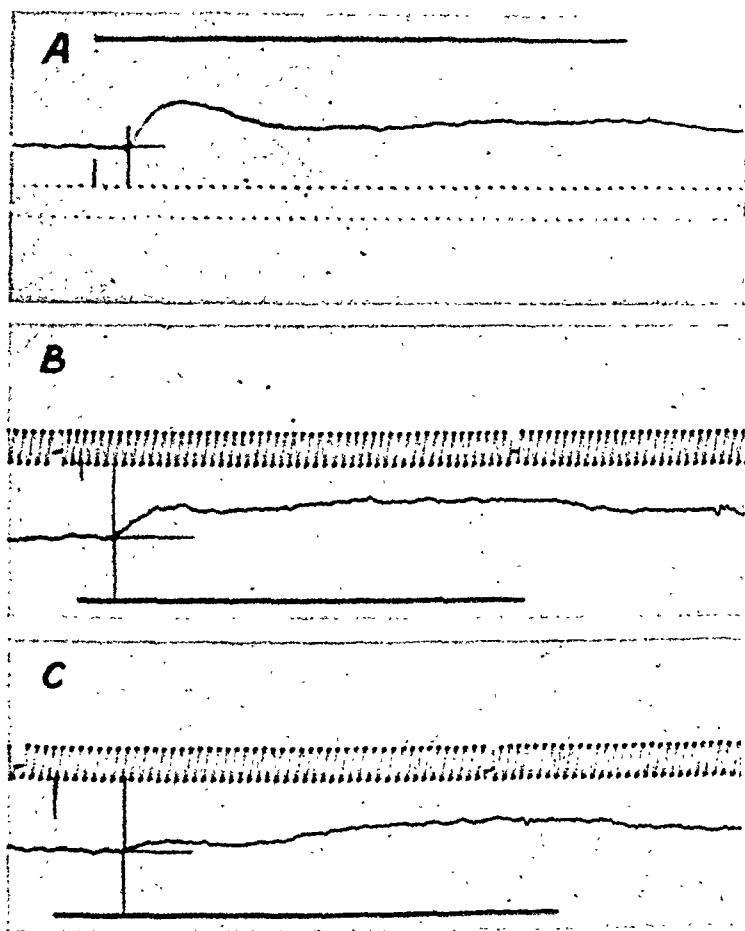


Fig. 23. Oscillograph records of retinal action potential of human eye at light intensities 1 (A), 1/10 (B), and 1/100 (C). Standard conditions.
Time in 1/50 sec.

obtained at intensity 1, shows no sign of any negative *a*-wave. After a latency of approximately 60 msecs, the *b*-wave rises straight from the unchanged base line. A slight secondary rise (*c*-wave) is visible. At the cessation of light, the effect falls slowly towards the base line without giving any noticeable positive off-effect. Fig. 24 A shows the retinal response to intermittent light at a low

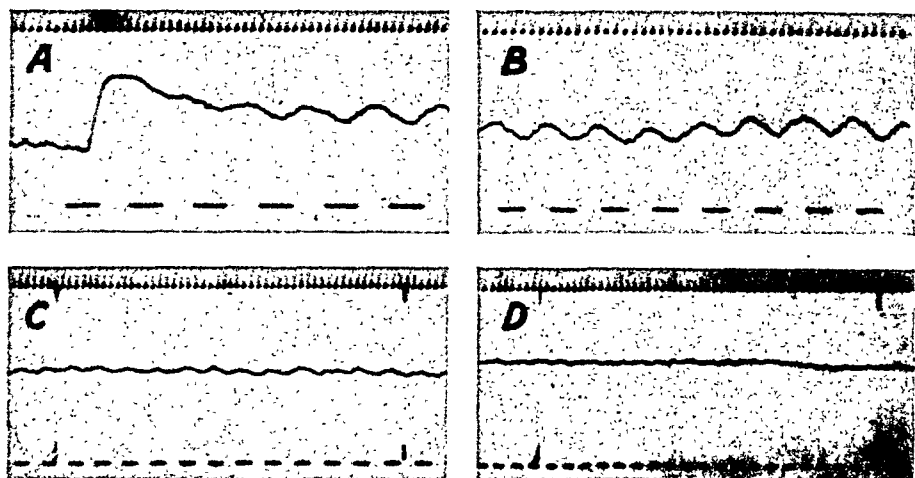


Fig. 24. Oscillograph records of human retinal action potential. Intermittent light at about 6 (A), 8 (B), 12 (C) and 20 (D) flashes per sec. Diameter of stimulating disc about $5^{\circ} 30'$ of visual angle. Time in $1/50$ sec.

rate of alternation. After the initial *b*-wave, potential ripples, such as "subliminal *b*-waves" follow on the general effect. Records in fig. 24 B—D illustrate the response at higher frequencies of the flickering light. Record 24 C still shows distinct waves which can no longer be identified in record 24 D. In this experiment as well as in others carried out for the sake of control and with the same light intensity and area, the potential oscillations could not be observed when the frequency exceeded 20—21 per sec., while the subjective fusion frequency in these experiments was 25—26 pr. sec.

The records in fig. 23 show the human retinal response at continuous illumination at three different intensities, corresponding to the three highest of the intensities used in the experiments on the blocking time. The different phases of the retinogram are not so clearly marked at the low intensities; in fig. 23 C they are still identifiable, but disappear entirely at lower intensities. As will be seen, the amplitude of the *b*-wave diminishes with decreasing light intensity (cp. GRANIT 1933, cat) at the same time as it shows a slower rise from the base line, a circumstance causing certain difficulties for the exact reading of the latency. The intensity used in fig. 23 C still allows, however, of a fairly accurate reading of the latency of the retinogram.

Table 2 shows the average values for the latency of the retinogram at the three different intensities (16 values at each).

Table 2.

Latency of the human retinogram at different intensities.

I n t e n s i t y	Latency of the b-wave in 1/100 sec.
1	5.8 ± 0.1
1/10	7.5 ± 0.2
1/100	12.1 ± 0.3

Finally, fig. 25 illustrates the simultaneous recording of the action potentials from the brain and from the retina at intensity 1.



Fig. 25. Simultaneous oscillograph records of occipital alpha blocking (upper cathode ray) and retinal action potential (lower cathode ray) at light intensity 1. Standard conditions. Time in 1/50 sec.

Discussion.

The results obtained confirm the observations of earlier authors concerning the general characteristics of the human retinal response to continuous and intermittent stimulation (GRANIT *et al.* 1933; KOHLRAUSCH 1931; GRÖPPEL *et al.* 1938 and others). What is most striking is the long latency and the relatively insignificant b-wave of the human retinogram. GRANIT *et al.* (1933) pointed out that these two facts might be explained by a relatively large negative component P III, whereas according to the same author, the absence of a visible a-wave does not support such an assumption.

According to the observations of GRANIT *et al.*, these investigations also show that the fusion frequency of the potential ripples seems to be lower than that of the subjective. With the illumination used, electrical fusion appears at a frequency value making

80 % of that of the subjective. This does not imply that the retinal and sensory fusion really *do* appear at different frequencies (GRANIT *et al.*, 1933). On account of the smallness of the potential waves, it is difficult to determine accurately the fusion point of the ripples on the retinal action potential.

The lower electrical frequency may, therefore, be explained on technical grounds, there being reason to suppose that the retinal frequency is not lower than the sensory (CREED, COOPER and GRANIT, 1933; CREED and GRANIT, 1933).

With regard to the absence of off-effect and the appearance of the response to flickering light, the human retina most closely resembles the E-type characterized by GRANIT (1935).

Fig. 25 shows the distribution of the total blocking time in a "pre-retinal" and "post-retinal" period with a simultaneous recording of the retinogram and the alpha blocking. As is evident from the discussion in part I (page 25), it is difficult to determine with accuracy the exact time for the start of the impulses judging by the retinogram. If the starting point of the human retinogram not only represents the point at which the positive component overcomes the invisible negative component but in reality the beginning of the *b*-wave, it is probable that the impulses start after a latency of 50—60 msecs. at intensity 1.

The long peripheral latency proved is scarcely surprising in consideration of the experiments on the motor reaction time at different sensory stimuli. Even in 1898 RICHET called attention to the fact that the relatively long reaction time at visual stimuli in relation to *e. g.* that of acoustic stimuli, is to be attributed to the slow course of the retinal processes. A comparison of the values obtained of the reaction time at acoustic and visual stimuli taken from works founded on more extensive investigations (*e.g.* RICHET, 1898; KOGA and MORANT, 1923; STARLING, 1930; MICHON, 1939) shows a difference of a magnitude which supports the functional significance of the long latency in the human ERG.

A comparison between the values obtained on the latency of the retinogram with those CRUICKSHANK (1937) gives on the latency for the local response from the human optic cortex would imply that the local cortical response "immediately" follows that of the retina. The local effect suggested by CRUICKSHANK (1937), must, however, be regarded as somewhat uncertain. In connection with these and earlier investigations (BERNHARD and SKOGLUND 1939) more than 50 individuals have been examined and

not in one single case has any distinct, definite local effect been proved.

If the impulses really start at the time when the retinogram begins, it is, however, very probable that the time between the beginning of the ERG and the "hidden" response from the optic cortex in the human brain is to be measured in milliseconds, as indicated by the conduction rate of nerve fibres in general (ERLANGER and GASSER, 1937), and judging by the investigations of the response of the optical cortex in warm-blooded animals (BARTLEY, 1936 b, rabbit).

It is therefore striking that the regularly appearing widespread form of cortical reaction consisting of the abolition of the alpha frequency appears so late. The alpha blocking does not appear until the *b*-maximum of the retinogram has been passed, indicating that a "central period" from the "hidden" local response to the blocking of the alpha waves goes up to more than 100 msecs.

It has already been pointed out (p. 68) that the prolongation of the blocking time at lower intensities will be referred to a prolongation of the latency of the peripheral processes. A comparison of the values for the latencies of the retinogram at those three intensities at which it has been technically possible to obtain retinograms suitable for analysis gives a further criterion for this.

The diagram in fig. 26 illustrates schematically for these three intensities the relation between the blocking time and that part which represents the latency of the retinogram. The whole column (both shaded and unshaded parts) represents at every intensity the value of the blocking time obtained from the diagram in fig. 20, while the shaded part gives the latency of the retinogram from table 2. As will be seen, the prolongation of the blocking time corresponds to the prolongation of the latency of the retinogram. It has previously been mentioned (p. 17) that the interval between the beginning of the retinogram and the starting point of the nerve impulses in experiments on animals was proved to be constant (ADRIAN and MATTHEWS, 1927 a, conger eel). Assuming that the same applies to the human being, the prolongation of the blocking time at the intensities tested is due to the prolongation of the latency of the peripheral processes.

The relation between the blocking time and the latency of the retinal response can for technical reasons only be investigated in limited intensities on humans. A comparison between the results

available on the human eye (table 2) and those on the frog in fig. 26 seem to suggest that the same relation will generally hold even for lower light intensities.

For the reasons stated above, the results can only be given with the accuracy allowed by the time unit (1/100 sec.) used. Any possible changes of the "postretinal" time of considerably less

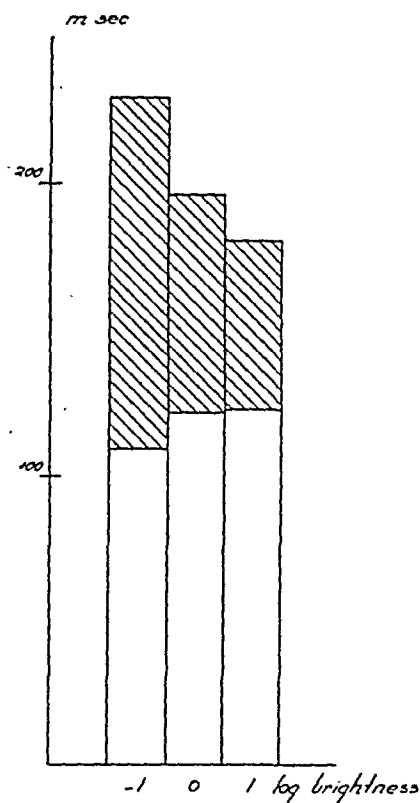


Fig. 26. Diagram showing the relation between blocking time (whole column, both shaded and unshaded part) and latency of the retinogram (shaded part) at light intensities 1, 1/10 and 1/100. Time in msec.

magnitude must consequently escape judgment. Nevertheless the experiments strongly support the assumption that the blocking time as a function of light intensity is mainly due to the peripheral processes.

In their previously mentioned experiments on the conger eel (see p. 8), ADRIAN and MATTHEWS (1927 a and b, 1928) proved that the latency of the optic impulses due to summative processes in the retina is a function of intensity, duration and area of illumination. It is also obvious from other observations that the block-

ing time closely reflects the changes in the latency influenced by the summative processes of the retina. Thus, it has been possible within certain limits to show that the blocking time is a function of the duration of the illumination (CRUICKSHANK, 1937). Again, it is evident from experiments carried out by the author in connection with the investigations described here that the blocking time is also a function of the area illuminated.

Relation between Blocking Time and Motor Reaction Time.

Technique.

The investigations were carried out under the experimental conditions already described (p. 61). The motor reaction and the blocking of the occipital alpha rhythm were recorded simultaneously. By means of a convenient contact in the right hand, the subject signalled when he saw the light. The contact was directly connected with the one beam of the oscillograph. In certain series of experiments the subject marked the moment he saw the light by means of a rapid bend of the right under-arm, and then the action potentials were registered from the biceps. Some 50 experiments were performed on 5 subjects. At least 16 values have been taken to form the basis of each average value in the table.

Results.

Fig. 27 A is a typical record with simultaneous registration of the blocking time and the motor reaction time at intensity $1/10$, which shows how the motor response at the marking with the hand contact takes place about 0.10 sec. after the blocking of the alpha rhythm. The reaction time thus registered is charged with a time interval due to the contact and its handling. In order to obtain further particulars as to whether the motor response really *does* take place after the alpha blocking, the muscle action currents were registered from the biceps when the subject, on seeing the light, quite slightly and rapidly bent the under-arm. Even this motor reaction, recorded without any mechanical loss of time, took place after the blocking of the occipital alpha rhythm (fig. 27 D). In the former case the difference of time between the motor reaction and the blocking time was on an average 80 ± 3 msecs, while in the latter it was 42 ± 4 msecs. for the same intensity.

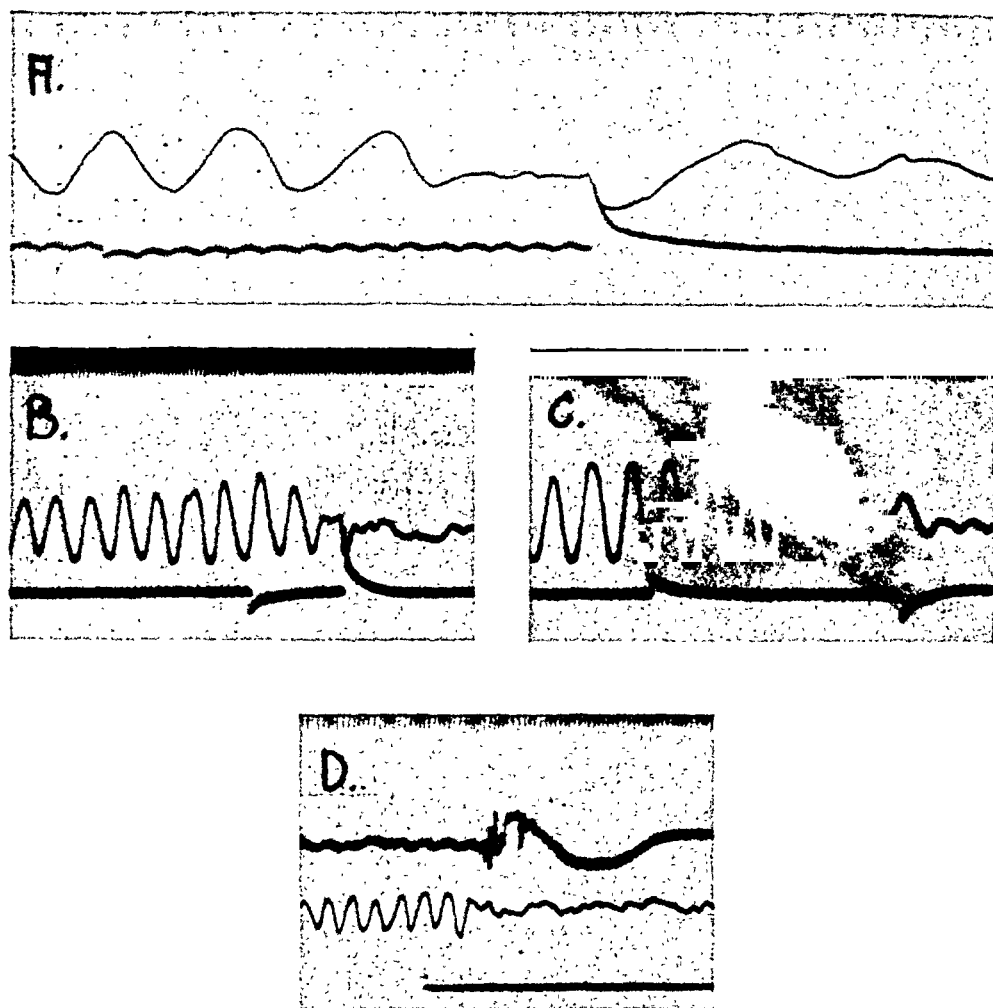


Fig. 27. Simultaneous oscillograph records showing the relation between the blocking of the occipital alpha rhythm and the motor reaction. A—C, hand contact (lower cathode ray); the onset of light is also marked on the lower cathode ray. Time in 1/100 sec. D, action currents from biceps (upper cathode ray). Time in 1/50 sec.

Intensities: 1/10 (A), 1 (B), 1/100000 (C), 1 (D).

Table 3.

The difference between blocking time and motor reaction time at different intensities.

Intensity	Difference in 1/100 sec.
1	8.0 ± 0.3
1/1000	7.6 ± 0.5
1/10000	8.3 ± 1.4

Table 3 shows the averages for the difference values between the blocking time and the motor reaction time (when marking with the hand contact) at three different intensities obtained on the person, whose blocking times at the same intensities are illustrated in table 1 (p. 65). As is seen, the difference between the motor reaction time and the blocking time is of the same magnitude at the three different intensities.

Discussion.

Based on the results already described, fig. 28 illustrates schematically the relation between the latency of the retinogram, the blocking time and the motor-reaction time at intensity 1. The

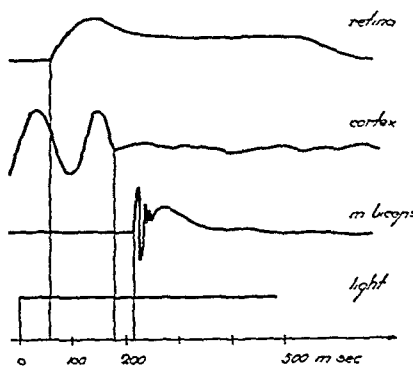


Fig. 28. Diagram showing schematically the different responses from retina and cortex in relation to motor reaction (action potentials from right biceps) to light stimulus (int. 1). Time in msec.

b-wave of the retinogram starts about 60 msec. after the onset of light, and the alpha blocking does not follow until after about another 120 msec. The motor response under the circumstances mentioned does not follow regularly until after the beginning of the alpha blocking. This latter circumstance was also pointed out by TRAVIS *et al.* (1937) when recording the action currents from the right extensor digitorum communis. The motor response taken from the biceps takes place about 40 msec. after the blocking of the occipital alpha rhythm.

The experiments above (p. 76) indicate that the blocking time at weaker light intensities is prolonged with the prolongation of the latency of the retinogram. The motor reaction time is also a function of light intensity (*e. g.* PIERON 1932), which is illus-

trated in table 3. DURUP and FESSARD (1936), having compared the prolongation of the blocking time and the motor reaction at different intensities, consider that the former is prolonged in comparison somewhat more than the latter at decreasing intensities.

This fact has not been able to be established in these experiments. At the simultaneous recording of the motor reaction time and the blocking time, the difference between them always proved to be constant in such a manner as is obvious from the typical experiments illustrated in table 3, which indicate that the reaction time at weaker stimulus intensities is prolonged in the same way as the blocking time.

Relation between Blocking Time and Perception Time at Different Intensities.

The fact that the reaction time as a function of intensity is prolonged parallel with the blocking time will show that the perception time undergoes a corresponding change; in other words, the experiments favour a time parallelism between the blocking time and the perception time.

In order to obtain further evidence for this, special discrimination experiments were carried out, in which a strong light stimulus was superimposed on a weaker one. In repeated experiments with varying intervals between the two light stimuli, the lowest value of time was determined between the onset of the two light stimuli, at which the subject stated that he could not perceive that the weaker light had preceded the stronger one. In such discriminating experiments the interval between the onset of the two light stimuli was determined when their perceptions fused to one. In connection with the discrimination experiments, the blocking time was determined for the two light intensities used.

Technique.

The experiments were carried out according to the conditions stated on p. 63. The blocking times for the two intensities to be used in the discriminating experiments were controlled on each occasion in about 10 records for each intensity. The exposure of the two consecutive light stimuli was carried out by means of the arrangements already described (p. 61) and the time between their onset was read on the records in 1/100 second units, the time

marker and the camera being used as a recording "chronoscope". The two light stimuli were tested repeatedly with varying intervals, and on each occasion the subject stated whether he had seen the weaker light precede the stronger or not. The interval between the onset of the two light stimuli was set to about the value at which the onset of the two stimuli began to merge into one single perception. The stronger light stimulus was in mostly maximal (intensity 1), whereas that of the weaker stimulus was varied. All told, about twenty experiments in total were carried out on different persons with the use of different intensities.

Results.

Table 4 illustrates a typical series of discriminations from an experiment in which the intensities 1/10,000 and 1 were used. The

Table 4.

(For further explanation see text.)

Interval between onset of two stimuli (intensity 1/10000 and intensity 1 respectively) in 1/100 sec.	The onset of the first stimulus perceived: +
40	+
32	+
28	+
22	+
22	
21	+
21	+
18	+
18	
17	
16	
10	
6	
6	

Average blocking time at intensity 1/10000	36/100 sec.
" " " " " 1	17/100 sec.
	Difference: 19/100 sec.

weaker light stimulus preceded the stronger at different intervals at the different exposures given in the left column. The plus sign in the right column indicates that the subject saw the onset of the dim light before that of the bright. When the interval between the onset of the two stimuli fell below 18/100 secs., that of the dim stimulus was not perceived. The table illustrates the often recurring fact that the subject sometimes stated that he did not

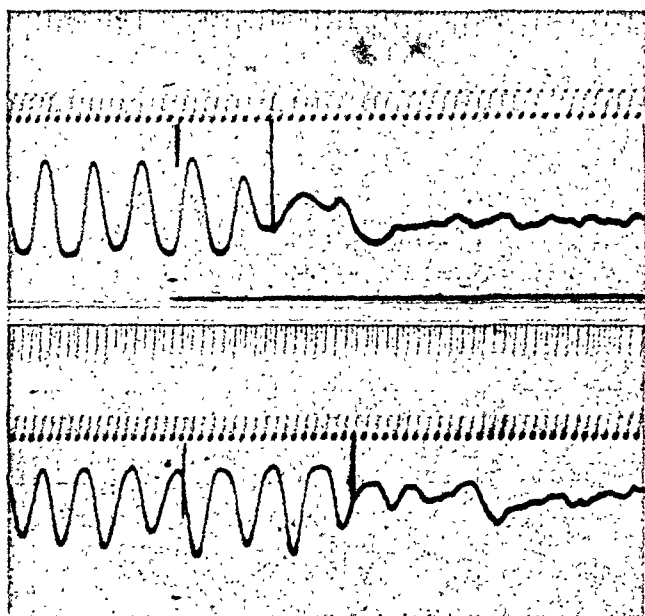


Fig. 29. Oscillograph records of the occipital alpha blocking to light at intensities 1 (A), and 1/10,000 (B) obtained in connection with the discrimination experiment illustrated in Table 4. (Subject nr 3).

see the dim light at intervals of a magnitude which in the majority of cases distinctly gave two perceptions. Such stray values as these are ascribed to varying attention, occasional blinking when the stimulus is lighted, etc. The value to be noticed in this connection is the lowest value of the time between the onset of the two stimuli at which the subject still perceived the onset of both stimuli, in this case 18/100 sec.

As will be seen, this value is of the same magnitude as that of the difference between the averages (see table 4) of the blocking times (cp. fig. 29) obtained in connection with the discrimination experiment for the two intensities used.

Table 5.

I	II					III	IV
Subject nr.	Blocking times in 1/100 sec. of stimuli at intensities:					Difference be- tween block- ing times of the two stim- uli in 1/100 sec.	Shortest interval for double perception in 1/100 sec.
	1/20000	1/10000	1/1000	1/100	1		
3	—	—	—	22	17	5	6
3	—	—	27	—	17	10	10
5	—	—	28	—	18	10	10
3	—	—	28	—	16	12	14
4	—	—	29	—	18	11	13
4	—	36	—	—	18	18	20
4	—	35	—	—	18	17	18
6	—	36	—	—	19	17	17
3	—	36	—	—	17	19	18
2	46	—	—	—	19	27	25
3	47	—	—	—	16	31	28

Further explanation see text.

Table 5 illustrates data from 11 typical discrimination experiments, in connection with which the blocking times for the different intensities used have been determined. The blocking times for the two light stimuli used in each series of experiments are given in column II. For the first, *i. e.* the weaker, the intensities 1/20,000, 1/10,000, 1/1000, 1/100 were used, while the stronger, *i. e.* the superimposed stimulus, remained the same (intensity 1) throughout. The difference of values is given in column III, while column IV gives the lowest intervals at which the subject in each particular case clearly perceived the onset of the two light stimuli. The table shows that in each separate case the difference between the blocking times of the two intensities is of the same magnitude as the discrimination value obtained. They are prolonged in the same way as the intensity decreases.

Discussion.

If a strong light stimulus is superimposed on a weak one, the onset of the two stimuli is perceived separately, if the interval

between them is sufficiently great as compared with their respective perception times (the time from the onset to the perception of light). If the interval between the onset is diminished, a value is gradually obtained, in which the perceptions of the two light stimuli merge into one. If a fixed time relation exists between the blocking of the occipital alpha rhythm and the perception of light, this value should be equal to the difference between the blocking times of the two intensities used. The results of the experiments above described firmly support this fact within the limits of accuracy that the time unit allows.

The values obtained in the discrimination experiments show (see table 5, column IV) how much longer the perception times are for the weaker light stimuli (intensities $1/20,000$, $1/10,000$, $1/1000$, $1/100$) than for the superimposed one (intensity 1). In the same manner the values in column III show how much longer the blocking time is for each lower intensity than for the highest. The agreement between the values in columns III and IV shows that the perception time with decreasing stimulus intensity is prolonged corresponding to the increase of the blocking time. Thus, the experiments show that a parallel of time exists between the perception of the light and the blocking of the occipital alpha rhythm caused by the light.

General Discussion and Conclusions.

The most obvious effect of the light stimulus on the human brain potentials is the blocking of the occipital alpha rhythm, some different interpretations of which (BERGER, JASPER, ADRIAN) have already been mentioned. The latency of this widespread form of cortical response to light *i. e.* the blocking time, is proved in experiments confirming earlier observations (DURUP and FESSARD, 1935 and 1936; CRUICKSHANK, 1937) to be a function of illumination intensity. This fact, as well as the circumstance that the blocking time has been proved to be a function of light duration (CRUICKSHANK, 1937) and area (own investigations), indicates that the blocking time closely reflects the changes of the peripheral latency which is influenced by the summative processes of the retina (ADRIAN and MATTHEWS, 1927 a and b; 1928). The comparative investigations concerning the latency in the optic impulses of the frog go still further to illustrate the time relationship between the peripheral and cortical events.

The most direct evidence that the prolongation of the blocking time is chiefly caused by the prolongation of the peripheral latency is obtained by recording the action potential of the human eye. Assuming that in human beings the interval between the retinal and nerve processes is constant (ADRIAN and MATTHEWS, conger eel), the prolongation of the blocking time within the intensity range tested (see p. 73) will be ascribed to the periphery, and there is no reason to assume that such is not the case concerning other intensities.

JASPER (1936) based his theory for the explanation of the blocking reaction (see p. 55) on the fact that the blocking time is a function of stimulus intensity, a quality that JASPER considers to be of cortical origin. The above investigations show, however, that the prolongation of the blocking time with decreasing stimulus intensities must be primarily due to the periphery. Neither have the slow potential changes maintained by JASPER (1936) been identified. Experiments carried out with the use of a direct-coupled amplifier do not point to any such regular potential changes. Both when using a D. C. amplifier and condenser coupled amplifier with a great time constant (JASPER, 1936), an absolutely steady base line cannot be relied upon when recording from the skin, a fact that considerably complicates the judgment of possible potentials with slow time characteristics.

Any definite local "evoked potential" has not been found in these investigations. There is, however, reason to assume from the above grounds that the local cortical response follows so closely upon the retinal that there must be an interval of approximately 100 msec. before the blocking of the occipital alpha rhythm takes place. The magnitude of this "central period" is somewhat surprising.

It may only be pointed out that the magnitude of this time corresponds approximately to the duration of an alpha wave. It has been proved that the alpha frequency is a function of age (*e. g.* LINDSLEY, 1936; SMITH, 1937; BERNHARD and SKOGLUND, 1939). Thus, at the age of 1 year it makes 5 waves per sec. and rises continuously thereafter, showing the most rapid increase in early childhood, and then, in later youth, gradually approaches the value of about 10 waves per sec, which is characteristic for the adults. BERNHARD and SKOGLUND (unpublished observations) found that the blocking time in different ages seems

to stand in relation to the duration of the alpha wave. They found that a one-year-old child, whose alpha duration is twice as long as that of a grown-up person, also shows a blocking time which is twice as long. The values in the ages ranging between seem to group themselves round about the curve for the duration of the alpha waves. A correlation of the results obtained by means of extensive investigations in the age developments of the reaction time in the case of different senses (KOGA and MORANT, 1923) indicates that the long blocking time of children's ages is a central phenomenon.

Whether a truly functional connection *does* really exist between the central part of the blocking time and the duration of the alpha wave, or whether they are two variables, independently related to *e. g.* the age, cannot be determined. This fact, which has also been pointed out by LINDSLEY (1938) is, however, worthy of note.

The investigations on page 80 show that there is a definite time relation existing between the perception of light — likewise a function of intensity — and the blocking of the occipital alpha rhythm caused by the light. The different mental phenomena following the presentation of a light stimulus (*e. g.* ERISMANN, 1935), discussed by psychologists, cannot be entered into here, suffice to say that in this connection perception time (Empfindungszeit, FRÖHLICH; Verarbeitungszeit, RUBIN) refers to the time from the onset of light until the time when the subject is sufficiently conscious of the light to be able to distinguish this onset from that of a following stronger light stimulus. The prolongation of the perception time with decreasing intensity in relation to the time for a higher intensity corresponds to the prolongation of the blocking time at decreasing intensity, as compared with the same higher intensity.

It was astronomers (*e. g.* MASKELYNE in Greenwich, 1795) that first began to take an interest in the value of perception time, and in order to correlate individual astronomical observations, they endeavoured to set up "a personal equation" (*e. g.* BESSEL, 1815). Several physiologists and psychologists subsequently tried to go more closely into the problem (*e. g.* EXNER, WUNDT, VON HESS, PULFRICH, HAZELHOFF, FRÖHLICH), and the reaction time measurements in the case of different sensory stimuli have attracted great interest (*e. g.* EXNER, KRIES, MARRAY, RICHET, among others); results were disappointing, however.

Among later methods for the determination of perception time, those worked out by HAZELHOFF (1923 and 1924) and FRÖHLICH *et al.* (*c. g.* FRÖHLICH, 1922, 1925, 1929; MONJÉ, 1925, 1934; VOGELSANG, 1925) are worthy of note.

FRÖHLICH's method is founded on a phenomenon which may be briefly described as follows. When a vertical light slit is rapidly moved horizontally and appears at the vertical edge of a screen placed between the movable light slit and the observer, the light slit is first seen at a certain distance from the edge in the direction of the slit movement, and not at the edge of the screen. The quotient of the slit displacement noticed by the observer and the value of the rapidity of the slit movement is given by FRÖHLICH as the measurement of the perception time. The motor reaction time is registered in the usual manner. FRÖHLICH *et al.* consider that this method makes it possible to divide the total motor reaction time to light into two parts, the "perceptual" and the "motorial".

Making use of FRÖHLICH's method, MONJÉ (1934, *cp.* VOGELSANG, 1925) found the perception time to be 33—40 msec. when using a light stimulus, the total motor reaction time of which is about 190 msec; he also came to the conclusion that the prolongation of the motor reaction time at lower stimulus intensities seems to be due to the prolongation of the perception time. MONJÉ's value of the absolute perception time appears to be somewhat low, considering the results already mentioned with regard to the latency of the retinogram at a light intensity with corresponding motor reaction time (see fig. 20 and table 2).

FRÖHLICH's method for the determination of the absolute perception time must, moreover, be regarded with a certain amount of scepticism on account of the criticism expressed from various quarters.

WIRTH (1927) pointed out the improbability of limiting the two given parts in the motor reaction time so accurately. RUBIN (1930 a and b) also criticized FRÖHLICH's method, and considers it incorrect to regard the slit displacement and the perception time as proportional magnitudes. ERISMANN (1935) finally discussed the problem thoroughly, both from a physiological and psychological point of view. He calls attention to the fact that the differences (*c. g.* for different light intensities) of perception time are possible to determine, but denies the possibility of the absolute determination of perception time as a whole, and raises strong objections to the methods of HAZELHOFF and FRÖHLICH.

Thus, an examination of recent literature gives the impression that the contradictory opinions are the outcome of attempts to solve a problem which is perhaps beyond the bounds of reason. As a matter of fact the question arises whether it really is possible to establish the absolute value of the perception time (see *e. g.* ERISMANN, 1935).

In face of what has just been said, it is clear that the results of these experiments do not allow any conclusions to be drawn regarding the relation between the blocking time and the absolute perception time, and consequently the causal relation between the blocking reaction to light and the perception of the light must be considered to be beyond discussion.

The results are nevertheless of interest, for they point to the fact that an intimate time relation exists between the electrical response of the cortex elicited by a light stimulus, and the subjective perception of light.

Summary.

1. Time characteristics of the electrical responses from the retina and the brain to light stimuli have been studied in humans. The electrophysiological data obtained have been correlated with sensory phenomena following light stimuli.

2. Confirming earlier observations, the investigations show that the latency of the blocking of the occipital alpha rhythm, *i. e.* the blocking time, is a function of light intensity.

3. The results point to a regular monophasic course of the curve representing the blocking time as a function of log brightness, similar to that representing the latency of the action potentials of the optic nerve as a function of log brightness obtained on frog.

4. The latency of the action potential of the human eye has been studied at different intensities, at which it has been technically possible to obtain retinograms suitable for analysis. The experiments strongly support the assumption that the prolongation of blocking time is mainly due to the prolongation of the peripheral processes in the retina.

5. Simultaneous records of the blocking reaction and the retinogram show the interval between the beginning of the retinogram and the commencement of the alpha blocking, the magnitude of which suggests a "central period" of more than 100 msec.

The approximate correspondence between this period and the duration of the alpha wave has been discussed.

6. Simultaneous recording of the blocking time and the motor reaction time show that the motor response follows after the blocking of the occipital alpha rhythm. The prolongation of the motor reaction time with decreasing intensity corresponds to that of the blocking time.

7. Special discrimination experiments have been carried out, in which a strong light stimulus at varying intervals was superimposed upon a weaker one. The interval between the onset of the two light stimuli, at which their preceptions fused to one, was proved to be of the same magnitude as the difference between the blocking times of the two light intensities used. The results point to the fact that a certain time relation exists between the perception of light and the blocking of the occipital alpha rhythm caused by the light.

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From the Physiological Department of the University of Lund,
Sweden.

ON THE
CITRIC ACID METABOLISM
IN MAMMALS

BY

JOHAN MÅRTENSSON

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Preface.

This investigation has been conducted at the Physiological Institute of Lund, and was commenced in 1937. The Head of the Institute at that time, Professor TORSTEN THUNBERG, introduced me to this field of inquiry and provided me with exceptionally good working facilities. Since then he has encouraged my work with the greatest interest and kindness, for which I tender him my respectful and hearty thanks.

To the present Head of the Institute, Professor GEORG KAHLSON, I proffer my sincere thanks for the favourable working facilities I was still allowed to enjoy and for his interested help.

To Professor GUNNAR AHLGREN I also owe my cordial thanks for kind help and for the instructive years of work I had at the Pharmacological Institute.

I also thank Miss MARGARETA BRORSSON for her good technical help in the laboratory, Mrs. KARIN WEBER-BERG for help with the preparation of the manuscript, and Mr. BERT HOOD for the translation of my manuscript.

The investigation has been conducted with the aid of generous financial assistance from the *Nordisk Insulinfond*, for which I am deeply grateful.

To my wife, who carried out all the exacting work connected with the citric-acid estimations and assisted me with the animal experiments, I owe my warmest gratitude for her great, self-sacrificing work and her good support throughout the entire investigation.

Lund, in May, 1940.

Johan Mårtensson.



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Introduction.

The important part played by citric acid in the intermediary metabolism of the body has been brought out with ever increasing sharpness in the investigations of recent years. These investigations have been chiefly based upon *in-vitro* experiments on minced tissues or enzyme preparations from these tissues. The present work is mainly an experimental inquiry into the normal *in-vivo* metabolism of citric acid in the mammalian body. In this work full advantage has been taken of the increased facilities offered by THUNBERG's enzymochemical method for the micro-estimation of citric acid. The inquiry has also been extended to some experimentally induced pathological conditions, with a view to elucidating the mechanism behind those changes in the citric-acid content of the serum which have been found in various morbid conditions in man, especially in injuries to the liver parenchyma, and which are utilized in the differential diagnosis of hepatitis and obstructive jaundice (SJÖSTRÖM, 1937).

The work was begun in October, 1937. As frequent discussions of the citric-acid question were appearing in the physiologico-chemical literature, I considered it necessary to publish a preliminary account of the results I had obtained (MÄRTENSSON, 1938), this being followed the next year by an investigation of a special problem attracting interest at the time (MÄRTENSSON, 1939). These results have been further corroborated by later experiments and the work has been extended to embrace new fields of inquiry. The experimental material is given here, on the whole, in the order the problems presented themselves in the course of the work or as was prompted by publications from other centres. Experiences and experiments bearing on methodological questions have been brought together in a special chapter. For reasons of space I have abstained from giving a review of the citric-acid literature, this having been done a few years ago (SJÖSTRÖM, 1937; GRÖNVALL, 1937).

Recent as well as earlier literature relating to problems dealt with here are reviewed in connexion with the respective experiments. A more general review of the occurrence, conversion and significance of citric-acid in the body has also been recently submitted by the author (MÅRTENSSON, 1940).

CHAPTER I.

Contribution to Thunberg's Method of Estimating Citric Acid.

THUNBERG published his enzymic method for estimating citric-acid (abbreviated "Ci", which also refers to citrate) in 1929, and gave a detailed description of it in 1933. Since then all the investigators who have made considerable use of the method have contributed to making it more exact or more practical for various purposes. (ÖSTBERG, 1934; LENNÉR, 1934; SCHERSTÉN, 1936; SJÖSTRÖM, 1937; and GRÖNVALL, 1937.) In this work I have as a rule used the method such as it has been elaborated by this accumulated experience. I submit here my own experience of the method and my tests bearing on methodological questions only in so far as they offer something new of practical interest or as they are likely to assist in clearing up some previously unclear aspect. Further, reference has been made to some recent Ci studies that have given results important for the enzymic method of estimating Ci.

The Citrate Curve.

In the following account it is assumed that the details of the method are known. In the discussion on the so-called Ci-curve the terminology usual in the Ci literature is used: the phosphate extract of cucumber-seed, the "enzyme solution", does not contain only Ci dehydrogenases but also other dehydrogenases and their substrates, the "spontaneous donors"; hence a distinction can be drawn between "Ci activity" and "spontaneous activity". The citrate curve consists of the "level line" and an "ascending leg", which meet at the "contact point". The curve for the unknown solution, the "X curve", is compared with the "standard curve".

SJÖSTRÖM (1937) has submitted the citrate curve to an experimental analysis, in which he shows the great bearing the spontaneous donors have on the appearance of the curve. It

is however not clear from his account that *the form of the Ci curve is entirely* (except in the spontaneous-decoloration tube) *determined by the co-action of the Ci activity and the spontaneous activity.* And his statement that, medially to the contact point, the spontaneous donors "steal" most of the methylene blue (MB), leaving only small fractions at the disposal of the Ci, gives an inaccurate idea of what happens. On the contrary, the fact is that proportionally to MB there is a shortage of Ci there, so that the latter is exhausted rather early and the Ci dehydrogenase clearly cannot decolorize more than a part of the MB. The rest is dealt with by the spontaneous activity, and the decoloration-time becomes longer the less Ci the tubes contain. It may be said that the ascending leg of the curve gives an inverse, magnified picture of the amount of Ci in the tubes medial to the contact point. *The height of the level line is determined by the Ci activity and spontaneous activity jointly.* The position of the contact point is laterally determined by the *ratio of the Ci activity to the spontaneous activity*: when spontaneous activity is relatively feeble the Ci dehydrogenase is responsible for almost the whole of the MB-decoloration, a larger amount of Ci is used up for this, and the contact point lies further laterally. *The slope of the ascending leg, also, is determined by the ratio the Ci-activity bears to the spontaneous activity.* As in Ci determinations the same enzyme is used both for the solution with unknown Ci-content and for the standard solution, the spontaneous activity is equal in both cases, and it is consequently *the Ci-content of the unknown solution which entirely determines the form of the curve relatively to that of the standard curve* (provided the solution is a pure Ci one or so diluted that other disturbing substances do not affect the reaction).

According to the above account the two straight legs of the Ci curve should meet at a contact point. But actually *the contact point is almost always more or less non-existent*, since one of the legs merges arcuately into the other. This is because the Ci-activity successively diminishes (under the law of mass action) after the Ci has come below the optimum concentration for the enzyme. The level-line does not start at that point in the tube series where the quantity of Ci is just in excess of the MB, but where the Ci-excess is so large that the amount of Ci required for the reduction of the MB does not bring the Ci-concentration below the optimum. In the usual arrangement of the experiment 5 γ (micrograms) of Ci correspond to the added quantity of MB, 10 γ , (in 2 cc.) but the optimum con-

centration is probably a little higher. According to DANN (1931), the Michaelis constant (the concentration for the semi-maximum conversion according to DAVIES and QUASTEL, 1932) for Ci-dehydrogenase from cucumber-seed is $8 \cdot 10^{-5}$ Mol. at 35° C, but his computation is doubtless somewhat uncertain. According to ADLER, v. EULER, GÜNTHER and PLESS (1939), this constant for isocitricodehydrogenase from heart muscle is less than $1.25 \cdot 10^{-5}$ Mol. and it is probably of the same order of magnitude for the cucumber-seed extract. Another reason for the ascending leg not being straight but curving upwards is that the spontaneous activity gradually declines in the course of the experiment, this, too, being due to the concentration of the substrate gradually falling below optimum.

Here the fact has been ignored that the so-called citricodehydrogenase is actually more complex than was known at the outset. WAGNER-JAUREGG and RAUEN (1935) showed that isocitric acid is also dehydrogenated by cucumber-seed extract, even more rapidly than Ci; hence it may be an intermediate product in the breakdown of Ci. Later on, MARTIUS (1937, 1938) submitted good evidence that the breakdown of Ci is initiated as follows: Ci is first deprived of water by a hydratase (similar to fumarase but not identical with this), which has been called aconitase, and is converted into the unsaturated *cis-aconitic acid*. This takes up water again and forms *isocitric acid*, which is afterwards dehydrogenated by the isocitricodehydrogenase. Explained on these lines the reaction accords better with the general view of dehydrogenation reactions, whereas a direct attack on Ci would have been difficult to explain. THUNBERG (1929) conceived of an initial rearrangement in such manner that water was taken up with formation of an ortho-acid, which could then be attacked by the dehydrogenase. Thus the enzyme system consists of *aconitase* and *iso-citricodehydrogenase* along with *co-dehydrase II* (ADLER, v. EULER, GÜNTHER and PLESS, 1939) and *flavoprotein* (WAGNER-JAUREGG and RAUEN, 1935).

ANDERSSON (1933) found an elevated Ci-dehydrogenation to result from addition of Co-enzyme, something that SCHERSTÉN (1936) was unable to confirm. The different results obtained by these investigators may be due to their Co-enzyme preparations having had Co-dehydrase II admixtures of different strengths, co-enzyme being according to ADLER and others (1939) completely inactive as against iso-citricodehydrogenase from animal tissues as well as from higher plants and yeasts.

The objection might therefore be raised to the enzymic method of estimating Ci that the rate of decoloration is not

determined by the iso-citric dehydrogenase but by the amount of Co-dehydrase II and flavoprotein. The cucumber-seed extract admittedly contains a certain amount of these co-enzymes, although I have not yet had an opportunity of testing whether they occur in optimum quantity. Otherwise the results might be influenced if these co-enzymes were supplied with the fluid to be tested in such quantity that the decoloration-time was thereby accelerated. According to v. EULER and SCHLENK (1939), co-zymase is present in the blood almost exclusively in the corpuscles. Co-dehydrase II is present in less quantity than co-zymase. And, moreover, as it is rather soon destroyed (through phosphatase activity) if the blood is allowed to stand, the risk of getting erroneous values through adding co-dehydrase II with the blood serum ought to be very small. With regard to the flavoproteins, of which the diaphorase¹ is primarily concerned here as the co-enzyme dehydrogenase occurring in the animal body (DEWAN and GREEN, 1937, ADLER, v. EULER and HELLSTRÖM, 1937, STRAUB, 1939, quoted from DIXON, 1939), this seems to be present in all animal tissue, but I have not been able to find any reports as to its presence in blood serum. An assurance that the presence of co-enzyme in the fluid to be tested cannot cause an error of any consequence in the Ci-estimation is offered by the fact that *the level of the X-curve is never found to lie with certainty below that of the standard curve*: this ought to occur if the tested fluid contains co-enzymes and the enzyme solution has not optimum concentration from them.

Martius' decomposition scheme has been confirmed by BREUSCH (1937) and ADLER et al. (1939), who find that in experiments with Ci the decoloration-time becomes shorter if Ci is incubated with the enzyme for some time before the other components are added; during this time the conversion to isocitric acid takes place. I have repeated the experiments with cucumber-seed extract without finding any shortening of the decoloration-time if Ci had stood with the enzyme under anaerobic conditions before the addition of MB. This applied to both Ci-solution and serum. A difference of some importance might be conceivable if the Ci and isocitric acid in the serum are in equilibrium from the start, whereas in the case of a pure Ci-solution this equilibrium is not established until after addition of the enzyme.

ANDERSSON (1938) has questioned whether the Ci added in the ex-

¹ Diaphorase II according to ABRAHAM and ADLER (1940), who consider that they have demonstrated the existence of two diaphorases.

periments really acts as a substrate. He thinks that it may just as well be interpreted as an activator after the fashion of the C_4 -dicarboxylic acids, and is inclined to see a proof of this in the small Ci -amounts required for the decoloration. It has probably escaped his notice that only very small amounts of MB are concerned as well; actually about equimolecular quantities of Ci and MB are involved, as THUNBERG already pointed out in 1929.

The Enzyme Stability and Spontaneous Donors in Cucumber-seed Extract.

In preparing the cucumber-seed extract I have, on the whole, followed SCHERSTÉN's (1936) directions, i. e. adopted long-time extraction in the refrigerator. However, I have devoted some experiments to the question of what takes place during the so-called self-purification of the enzyme extract and to the question of the spontaneous donors and the stability of the enzyme.

SCHERSTÉN's experiments show that Ci -dehydrogenase is stable for a long time if the enzyme is ice-cooled, and that the spontaneous-donor systems are far less stable. Respecting the conditions at higher temperatures his account does not give definite information. SCHERSTÉN presumes that the lowered spontaneous activity associated with long extraction-time may be due to an exhaustion of the spontaneous-donor systems.

If the same enzyme preparation is tested after extraction-times of different length it is found that the *spontaneous activity declines successively, while the Ci -activity keeps fairly constant (Fig. 1).*

This decline in the spontaneous activity is due to exhaustion of the spontaneous-donor substances, not to inactivation of the dehydrogenases attacking these substances.

Experiment 31. 1. 40. Extraction with M/15 phosphate buffer of pH 7.38 in room temperature, 30 min., whereupon the extract was centrifuged and tested for activity: spontaneous decoloration 15 min.; with 10 γ Ci 13 min. Part of the solution, portion I, was boiled, filtrated, and diluted to its original volume. The rest of the enzyme solution was allowed to stand 90 min. at room temperature, whereupon another part, portion II, was boiled, and the remaining enzyme was used for the experiment; the tubes were charged with Ci , portions I and II respectively. Decoloration-times: See Fig. 2.

Portion I contained the stable donor substances in the enzyme with a spontaneous decoloration-time of 15 min., portion II the donors with a spontaneous decoloration of 130 min. That the dehydrogenase activity

was none the less in the same good condition could be seen from the fact that an addition of 1 cc. of portion I gave the 15 min. decoloration-time again.

SCHERSTÉN (1936) found that the activity of the enzyme solution becomes less if the extraction-time is reduced to 15 min. or less, while the spontaneous-donor content becomes proportionately larger. The cause of this was assumed to be that the spontaneous donors enter in solution more readily than the enzyme, or possibly that during the longer extraction-time the spontaneous donors are used up more. That this latter assumption is correct was shown by the preceding experiment.

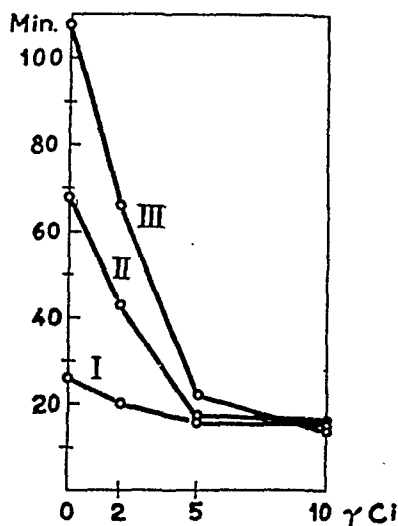


Fig. 1. Cucumber-seed extract with M/20 K_2HPO_4 is divided into three portions extracted in room temperature during 1, 2 and 3 hours respectively.

But the former hypothesis is undoubtedly correct as well, it being possible to obtain the same type of enzyme solution even with *long-time* extraction if the cucumber-seeds are pounded and stirred less thoroughly: then the enzymes — Ci-dehydrogenase as well as the others — dissolve with difficulty, whereas the donor substances enter readily in solution, in which they are only slightly oxidized on account of the low enzyme activity.

Experiment 31.10.38: Extraction with M/20 K_2HPO_4 one hour in refrigerator and 45 min. in room temperature.

Decoloration-times:

Ci in tubes	0	2	3	4	5	6	8 γ
High degree of stirring	44	25	20	14	12	12	11 min.
Low " " "	23	21	20	20	20	20	"

Experiment 14. 10. 38: Extraction with M/20 K_2HPO_4 overnight in refrigerator.

Ci in tubes	0	2	3	4	5	6	8 γ
High degree of stirring	—	—	61	49	22	14	11 min.
Low „ „ „	16	12	10	8	8	8	8 „

The experiments reveal the technically important fact *that the stirring of the cucumber-seed extract must always be carried equally far if standard curves of a constant form are to be obtained.*

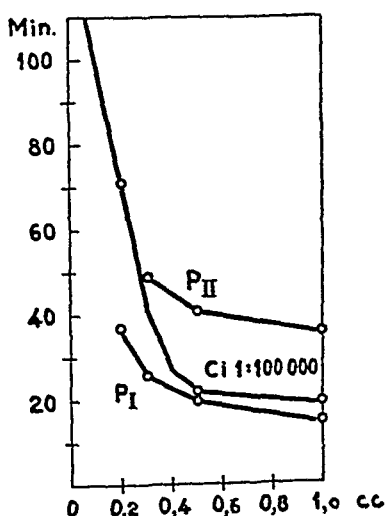


Fig. 2. MB decoloration by donor substances in boiled cucumber-seed extract (see text).

From some tests of the enzyme stability at 35° (the temperature at which Ci estimations are usually made), SCHERSTÉN infers that the Ci-dehydrogenase is more rapidly inactivated if the spontaneous donors are reduced too much, i. e. the spontaneous-donor system is thought to have a protective influence on the Ci-dehydrogenase. In one case the enzyme was inactivated so much in 30 minutes that the decoloration-time for 6 γ Ci rose from 16 to 70 minutes. This, however, might simply be explained thus, that this quantity of Ci was at first (when spontaneous activity was high) just in excess relative to the quantity of MB but was afterwards (when spontaneous activity fell) insufficient; in such cases there can be rapid and great changes. *The Ci-dehydrogenase activity in the cucumber-seed extract remains a long time unchanged even at 35° and even if the spontaneous activity is reduced to a minimum.*

Experiment 9.2.39. Extraction from cucumber-seed for 13 hours in refrigerator with M/20 K_2HPO_4 . After being centrifuged the enzyme solution was ice-cooled 30 minutes and a first series was then set up, whereupon the enzyme was placed in a 35° water-bath. At intervals of 30 minutes other series were then set up. The decolorizing times are given in *Fig. 3*.

The experiment also gives an idea of the successive decrease in intensity the spontaneous activity always undergoes during

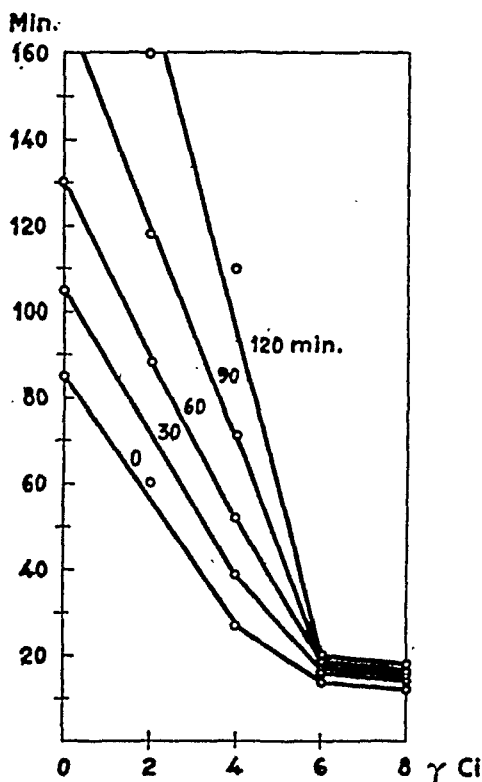


Fig. 3. Change in citrate-curve resulting from storage of the enzyme at $35^\circ C$.

the decolorization. When the spontaneous reduction time, as in the first series, is about 90 minutes, the dehydrogenation intensity at the end of the decolorization should be only half that at the start (since the spontaneous decolorization takes double the time after the enzyme has had a temperature of 35° for 90 min.). Thus the intensity of the spontaneous activity at the beginning of the experiment cannot be calculated simply as the inverted value of the decoloration-time; it is considerably greater. The increased decoloration-time for the un-

doubtedly optimum Ci-quantities in the above experiment (from 12 to 18 min.) can therefore no doubt be attributed to diminution of the spontaneous dehydrogenation.

Doubling the spontaneous activity by adding twice the amount of enzyme without other addition in no wise means merely a halving of the decoloration-time, but a considerably greater shortening of this time. In *experiment 2. 2. 40* the spontaneous decoloration took 87 min. with 0.5 cc. of enzyme, but 20 min. with 1 cc. of enzyme, i. e. less than one-fourth. In *experiment 31. 1. 40.* it was 130 min. with 0.5 cc. of enzyme, 41 min. with an addition of 0.5 cc. of boiled enzyme extract (i. e. without enzymic activity, only containing the spontaneous donors), thus less than one-third. If one adds a substance that accelerates decoloration, one is therefore apt to ascribe to this substance too great a participation in the increased dehydrogenation activity. THUNBERG (1936) has given a thorough analysis of the factors to be observed in calculations of the kind in question. He has none the less used a "minute decolorization value" derived from the spontaneous-decoloration time, pointing out that the calculation is not quite correct but may yet be of use in practice.

An oxidative process is thus constantly going on in the enzyme solution, but it is of very low intensity if the solution is kept effectively cooled down. (That the enzyme solution, as earlier investigators have pointed out (LENNÉR, SCHERSTÉN), is most labile during the first 30 min. after centrifugation, is probably due most to the fact that the solution has not had time to become properly cooled down.) None the less, if for some reason a tube fails at the beginning of a large series, a certain amount of error is to be expected if a fresh tube is loaded and evacuated at the end of the series and inserted in the place of the old one.

Should the enzyme solution be found to be "too strong" on account of excessive spontaneous activity, *a suitable citrate curve will not be obtained by diluting the enzyme, since this also reduces the Ci-activity, but by keeping the solution at room temperature for some time.* Still, long-time extraction in a refrigerator probably offers better prospects of getting equivalent enzyme preparations on all occasions than does short-time extraction at room temperature.

If the spontaneous activity is very high, the entire Ci-curve becomes a horizontal line, which means that the decoloration-time cannot be shortened by addition of Ci. This can scarcely be interpreted otherwise than that *the enzyme solution itself contains Ci in optimum quantity from the start, which must be dehydrogenated before the typical citrate-curve can be obtained by adding Ci.* This is by no means unreasonable, for it merely presupposes that the enzyme solution contains 20 γ

of Ci per cc., that is to say, that the Ci-content of the cucumber-seeds is about 0.02 %. The great activity of the Ci-dehydrogenase quickly reduces the content to suboptimum concentration, 10 γ of Ci per cc being probably converted in 10—15 minutes at 35°. (The previously described experiment with addition of boiled enzyme agrees well with this: boiled enzyme having a high content of spontaneous donors gives a fairly typical citrate-curve, Fig. 2). This was later confirmed in direct estimation by the pentabromacetone method: one phosphate extract prepared in the usual way was analysed after it had been centrifuged and found to contain 17 γ per cc., in another the content was 20.4 γ per cc., which had fallen to 10.6 γ per cc. after the enzyme had been kept at room temperature for 90 minutes. Direct extraction from the cucumber-seeds with trichloroacetic acid yielded a quantity *equivalent to 350 γ of Ci per gm. of cucumber-seed* (in another case about 400 γ per gm.) This may mean that Ci plays some part in the metabolism of the seeds, and it affords a reasonable explanation of the abundant Ci-dehydrogenase content of the cucumber-seeds.

This condition also explains why the spontaneous activity, if it is very powerful, cannot be paralysed by ZnCl_2 (SCHERSTÉN, 1936): if it depends on Ci, it can obviously only be paralysed by ZnCl_2 when the latter is in such concentration as will also inhibit the Ci-activity.

With reference to the other substances that are probably contained as spontaneous donors in the cucumber-seed extract, I have conducted some experiments on the dehydrogenation of malic and hexose-diphosphoric acids (Preparations used: l-malic acid, British Drug Houses, and Hexosediphosphorsäure Calcium rein "Bayer"). The decoloration-curves for these substances are in good agreement with those given by THUNBERG (1929 a and b). For hexose-diphosphoric acid the "level" is lower than for Ci, for malic acid generally somewhat higher than for Ci. But for these substances there is, compared with Ci, quite a different proportion between the plainly active concentration and the optimal one: a series of tubes loaded with a solution of these substances in the same quantities as are used for Ci gives a considerably more "horizontal" decoloration-curve.

Experiment 21. 12. 39:

Ci	2		3		4		5		7
Malic acid		0.2		0.3		0.4			0.5 mgm.
Decoloration-time	59	55	42	47	28	43	20		40 min.

Experiment 2. 2. 40:

Ci	2		5		10	7
Hexose-diphosph.		4		10		100
Decoloration-time	45	35	15	17.5	12	7.5 min.

These substances alone can therefore scarcely simulate Ci, though their admixture to a Ci-solution may cause a certain amount of error in the estimation by making the ascending leg of the curve less steep. So far as malic acid is concerned, however, so great a concentration is required that it can hardly occur other than in tolerance tests with malic acid. On the other hand, hexose-diphosphoric acid, even in the same concentration as Ci, can play a certain part, but when a large quantity of solution is added the decoloration-time will be shorter than that represented by the level of the standard curve. This ought to arouse suspicion, control tests with addition of monoiodoacetic acid can then decide the matter. However, hexose-diphosphoric acid is probably not present in the serum in demonstrable quantity, no more than it is in freshly taken mammalian muscle (DEUTICKE and HOLLMANN, 1939).

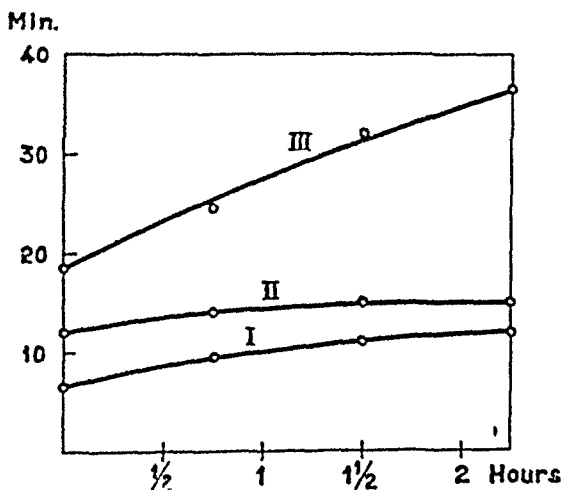


Fig. 4. Decolorizing intensity in cucumber-seed extract, stored at 35°, after addition of hexose-diphosphoric acid 0.1 mgm. (I), citric acid 0.01 mgm. (II), and malic acid 9 mgm. (III).

Some tests with enzyme that had been kept at 35° showed that the *intensity of the malicodehydrogenation declined in a*

much higher degree than that of hexose-diphosphoric and of especially the Ci dehydrogenation.

Experiment 3. 2. 40 and 5. 2. 40. Extraction from cucumber-seed with M/15 phosphate of pH 7.38 overnight. After the enzyme solution following centrifugation had been ice-cooled for 30 min., a first series was set up, whereupon the enzyme was placed in a water-bath at 35°. Other series were set up at intervals of 45 min. *Fig. 4* shows the decoloration-times (means of two experiments). A last series with enzyme that had been kept ice-cooled the whole time had the same times as the first.

The result need not imply that the malic dehydrogenase is more thermolabile than the other dehydrogenases, the inactivation being possibly also due to a lowered co-enzyme content. According to ANDERSSON (1938), for optimum activity the malic dehydrogenase requires a considerably larger co-enzyme content than the hexose-diphosphoric dehydrogenase, and hence any decrease of this content in the extract would primarily affect the malic dehydrogenation.

The Citrate Curve at different Hydrogen-ion concentrations.

ANDERSSON (1938) has studied the activity of Ci-dehydrogenase in cucumber-seed extract in phosphate buffers of different pH between 7 and 8. He found that the activity is maximum above 8 and declines very rapidly with falling pH, being entirely inhibited at pH 7.2; hence at lower pH added Ci should have no effect on the decoloration-time. ANDERSSON, on the basis of this finding, raises grave objections to the enzymic method for estimating Ci. In this he appears to rely on only one experiment with old enzyme extract and does not seem to have studied the literature on the subject.

DANN (1931) investigated the activity of the Ci-dehydrogenase in cucumber-seed extract (with optimum Ci-addition) and found the optimum at pH 7–10; below pH 6.5 the activity fell quickly, reaching minimum at pH 3.5. ADAMS (1931) found similar conditions for an aqueous extract of cucumber-seed: the difference in activity was small between pH 6.2 and 8.3, in more acid solution the activity was less and at pH 4.6 appeared to be altogether inhibited. ÖSTBERG (1931) published experimental series with the same decoloration-times within the range pH 7.15 and 7.55. He got some degree of difference when the pH was, on one hand, below 7 (9.5 min.), on the other hand, between 8.15 and 7.40 (6 min). Some workers have also studied the breakdown of Ci at different pH in metabolic ex-

periments: LANGECKER (1934) found in minced liver tissue no difference in Ci decomposition between pH 6.9 and 8.2. In similar experiments ISHIHARA (1938) found only a slight difference between pH 6.2 and 8.2. KREBS and EGGLESTON (1938) had good Ci metabolism in pigeon muscle at pH 6.8 in phosphate buffer.

For isoCi-dehydrogenase from heart muscle ADLER, v. EULER, GÜNTHER and PLESS (1939) got the same pH-curve as for other dehydrogenases: high activity between pH 7—7.5 and rapid fall below pH 6.5. They used, however, veronal-glycine buffer, having found that phosphate ions arrest the activity and that this arrest varies considerably with the pH: with phosphate buffer the curve has its maximum between pH 6 and 6.5, where the arrest is relatively small, and its minimum between pH 7 and 8, where the arrest is great.

I have repeated the tests on the sensitivity of cucumber-seed extract to pH fluctuations within the ranges concerned here. Like earlier workers, I found that *the activity of the Ci-dehydrogenase varies very slightly, if at all, within the range pH 6.6—7.6, whereas the spontaneous activity rapidly declines with falling pH and ceases almost entirely below pH 7.*

The following experiments may be adduced:

1. (13. 1. 40) Two cucumber-seed preparations, 13-hour extraction in refrigerator with M/15 Sørensen's phosphate buffer of different pH. (pH measured after the tests, after MB had been recoloured, with a quinhydrone electrode and audion valve potentiometer.)

Ci in tubes	0	2	5	10	7
pH 7.53	58	28	15	12	min.
pH 7.10	> 240	148	20	18	„

2. (22. 1. 40) Extraction from cucumber-seed with M/20 K_2HPO_4 at room T.; so spontaneous activity was very low. In tubes M/15 phosphate buffer of different pH. In every tube undoubtedly optimum Ci-quantity, 10 γ ; so the decoloration was practically speaking a measure of the Ci-activity.

pH	6.64	7.25	7.54
Decol. time	20	18	17 min.

3. (22. 1. 40) Extraction from cucumber-seed with M/20 K_2HPO_4 in refrigerator short time, great spont. activity. In each tube suboptimum amount of Ci, 3 γ ; so decoloration time was a relative measure of the spontaneous activity.

pH	7.00	7.12	7.28	7.34	7.71
Decol. time	89	55	48	43	36 min.

(Tests with unbuffered aqueous extract from the seed showed no complete spontaneous decoloration because the reaction was displaced to below pH 7 in the course of the experiment.)

4. (1. 2. 40) Extraction from cucumber-seed with M/30 phosphate pH 7.38. In the tubes M/15 phosphate to different pH. In each tube *l-malic acid* brought to M/75. Little spont. activity. (> 110 min.)

pH	6.85	7.07	7.18	7.32	7.42	7.56
Decol. time	31	27	25	22	21	18 min.

The pH curve for the malic acid does not argue against *part* of the spontaneous activity depending on a malic dehydrogenation.

5. (9. 2. 40) Overnight extraction from cucumber-seed with M/15 phosphate, pH 7.38. In tubes 80 γ hexose-diphosphoric acid and M/15 phosphate of different pH. Spont. decol. time 115 min.

pH	6.63	6.74	7.00	7.18	7.32	7.60
Decol. time	17	16	15	13	12	11 min.

The hexose-diphosphoric dehydrogenation is thus also sensitive to acid reaction. The latter has rather little influence when decoloration is rapid, but, if the spontaneous-decoloration time at alkaline reaction is already, say, 80 min., only a slight reduction in the activity is required for decoloration to cease altogether.

In respect of the spontaneous donors as well as Ci my experiments yield results directly opposite those of ANDERSSON's. With suboptimum quantities of Ci and strong spontaneous activity my tests give a pH-curve that is very similar to that ANDERSSON found for the spontaneous donors together with Ci. It is possible that in this test of his the spontaneous donors played the dominant part and that his erroneous result is due to this.

As, then, the spontaneous activity in the cucumber-seed extract is sensitive within a certain range to pH fluctuations, it is necessary to work with buffered solutions. Buffering in extracts made with K_2HPO_4 ought to be sufficient in most cases, but to be on the safe side I went over to extracting with M/15 phosphate buffer of pH 7.38 — it may be suitable to bring the hydrogen-ion-concentration within the range it usually has in blood-serum ¹.

¹ In my last experiments, in which cucumber-seed belonging to another delivery was used, it proved necessary to extract with phosphate of about pH 7.55 in order to get a suitably high spontaneous activity.

The possibility of inaccurate values arising from a change produced in the pH by added serum should *a priori* be negligible: firstly, the buffer-effect of the serum itself is not so great ($< 5\%$ of the whole blood's) secondly, the serum is mostly considerably diluted in the fluids concerned. And a pH-displacement would make itself the more noticeable, the higher up it occurred on the ascending leg of the curve but there the addition of serum — and with it the possibility of a pH-displacement — becomes progressively less. If, moreover, the ascending leg is "in line" with the spontaneous-decoloration time of the enzyme solution, there is in this, too, an assurance that the added serum has not altered the pH. Nor have I been able to ascertain any such at direct measurements.

The inactivation of heart muscle isocitricodehydrogenase by *phosphate ions*, demonstrated by ADLER *et al.* (1939) appears not to apply to the enzyme system in cucumber-seed extract. An increase of the phosphate concentration certainly gives a longer decoloration-time, but the retardation is of about the same order of magnitude as follows an increase of the NaCl concentration. Nor have I been able to find any difference in the intensity of the inhibition at different pH.

Experiment (15. 1. 40): Decoloration-time at pH 7.76 with different phosphate concentrations.

Ci in tubes	2	5	10	γ
Phosphate M/80	54	20	14	min.
„ M/40	57	25	16	„

Unless specially purified, aqueous extracts also contain some concentration of phosphate, since the cucumber-seeds themselves contain rather much phosphate: after the oil has been expressed, the residue contains 3.7%, reckoned as P_2O_5 (WEHMER, 1931).

ADLER *et al.* (1939) had also found that *manganese* or *magnesium* were necessary complements for isocitricodehydrogenase action. If this is the case with isocitricodehydrogenase from cucumber-seed, there are doubtless sufficient quantities of these substances in the extract, for no activating effect follows their addition in the concentration stated to be optimum.

Manganese (*Mn sulfur. Kahlbaum p. analys.*) was tested in concentration from M/5000 to M/250 and had no effect. Magnesium (*Mg sulfur. puriss. cryst. Merck*) was indifferent (or possibly feebly activating) in the low concentrations, but from M/500 it was inhibitive.

Experiment 5. 1. 40. Cucumber-seed extract with M/20 K_2HPO_4 . Decoloration-time with rising Mg:

Ci in tubes	3	5	8	7
MgSO ₄ 0	26	17	16	min.
„ 0.75 . M . 10 ⁻³	22	—	15	„
„ 1.5 „ „	24	17	15	„
„ 3.0 „ „	31	22	18	„
„ 6.0 „ „	44	30	26	„

ADLER *et al.* (1939) also claim that *monoiodoacetic acid* is a very powerful inactivator of heart muscle isocitricodehydrogenase: M/100 gives a 97 % retardation. This does not apply to the isocitricodehydrogenase in cucumber-seed extract. On the contrary, monoiodoacetic acid has proved to be a suitable addition for inhibiting the hexosediphosphoric dehydrogenase without inactivating the Ci-dehydrogenase. Even so high a concentration as M/40 produces only a 35 % retardation.

Experiment 22. 1. 40:

In each tube 10 γ Ci

Monoiodoacetic acid	—	M/1000	M/200	M/40
Decoloration-time	12	14	15	19 min.

Influence of Light on Rate of Decoloration.

The works of LEHMANN (1922), KRESTOWNIKOFF (1927) and v. EULER and KLUSSMANN (1934) show that increased light quickens decolorization in the Thunberg experiments. GRÖNVALL (1937) was the first to study the influence of light in enzymic Ci-estimations. He found that if light was kept from the tubes the decoloration-time was lengthened, while if artificial light was added to the daylight the time was shortened. This only applies, however, if MB or thionine is used as indicator, and not indigo trisulphonate, which is far less sensitive to light variations.

The great influence of light in Ci-estimating work is observed without special tests. On a clear, sunny day decolorization is very rapid, on a cloudy, rainy day it may even happen that tubes with a small quantity of Ci never become decolorized. At times the light varies very much in strength while an experiment is going on, which has a very disturbing effect. To avoid this element of uncertainty I found it simplest to *adopt entirely artificial lighting*: I could then retain MB as indicator, which is easier to read off than indigo trisulphonate. Control tests showed that the form of the curve is about the same in electric light as in daylight of the same intensity. This applies to both the standard curve and the X-curve, and hence the

same values are obtained if the estimation is conducted in strong light on one occasion and in less strong light on another.

Experiment 28, 30 and 31. 1. 1939. A series of estimations made in (1) daylight, (2) electric light, (3) daylight plus electric light. To eliminate effects from the change the enzyme is undergoing the whole time, the tests were carried out on three days in alternating sequence as regards the lighting. The series were then collocated, and the mean values of the decoloration-times are given in *Fig. 5*.

Experiment 10. 1. 40. Electric light. Distance from source of light in first series 100 cm, in second series 141 cm. The illumination is therefore double as strong in the former case as in the latter.

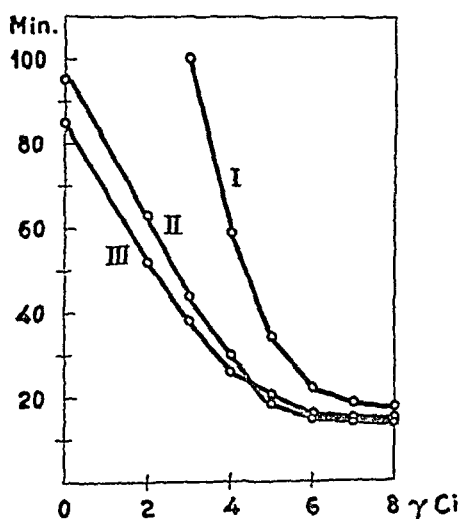


Fig. 5. The citrate-curve for different intensities of illumination: I Day-light, II Electric Light, III Day-Light + Electric Light.

Ci in tubes	0	2	3	4	5	6	7	8 γ
I	71	45	31	20	15	13	12	12 min.
II	87	58	43	27	20	16	15	14 „

When decoloration is relatively rapid a change in illumination does not cause so great changes in the decoloration-time, but if the latter is fairly long from the start a slight decrease in the lighting may result in failure of complete decoloration. A lengthening of the decoloration-time gives the inactivation of enzyme or coenzyme going on during the experiment a better opportunity of making itself felt.

The lighting arrangements were as follows: The estimations were carried out in a room entirely screened from daylight. The water-bath was illuminated by two ordinary Osram lamps (lum. effect: 100 decalumens).

These were attached to a stand behind and above the person managing the evacuations and readings, so that this work could proceed without the tubes being shadowed; illumination from behind is also less straining to the eyes. The distance from light-source to water-bath was at least 120 cm, the two lamps were 40 cm apart, while the series of tubes had a maximum range of 60 cm. In this way differences in illumination in different parts of the series can only be very slight and of no practical importance. By increasing or decreasing the distance it is a simple matter to vary the lighting intensity if decolorization is proceeding unsuitably fast or slowly. When, as is usual, samples are estimated twice with different enzyme preparations, they should be set up the second time in reverse order to the first so as to eliminate any illuminating differences or other irregularities in the routine work.

The form of the X-curve in relation to the Standard Curve.

Reference is made below to some aspects of the X-curve which seem in need of accentuation, not having received due attention in the Ci literature.

The method is intended for work on very small Ci quantities, so that disturbing substances in the test fluid can be neutralized by dilution. But with rising dilution the liability to errors in pipetting and reading-off is also increased. With the usual tube-loadings (0.2—0.3—0.4 cc. etc.) the error liability for tube 0.2 cc. is two and a half times greater than for tube 0.5. In the region in which the curve just bends round into the level-line, an insignificant variation of the decoloration-time may produce very great changes in the values. For this reason it is *safest to rely on the values obtained immediately above the bend of the curve*, as recommended by ÖSTBERG (1934); the liability to error subsequently increase the further up the curve one comes.

When a Ci-rich solution is being estimated, it must be diluted before the tubes are loaded, since in routine work one cannot pipette up smaller quantities than 0.1 cc. with any precision. Otherwise it is obviously of no consequence whether the diluting is affected before or at the loading of the tubes, provided use is made of the same diluting fluid (for instance, 0.2 cc. of an undiluted solution contains the same amount as 0.4 cc. of the same solution diluted 1:2). Excessive diluting, however, will restrict estimations to the upper, uncertain part of the curve, which spreads out over several tubes. Obviously

the usual type of curve cannot then be expected. Fig. 6 shows the theoretical course of the curve for different dilutions of the same Ci-solution. With a dilution of only 1:4 the curve does not extend down to the level-line. The curve 1:4 implies that the Ci quantities in the three top tubes of the 1:2 curve have been distributed over six tubes instead of three. Clearly the *relative* differences between the tubes are smallest furthest down the curve, and it may therefore be very difficult in practice to determine any difference in decoloration-time: one gets

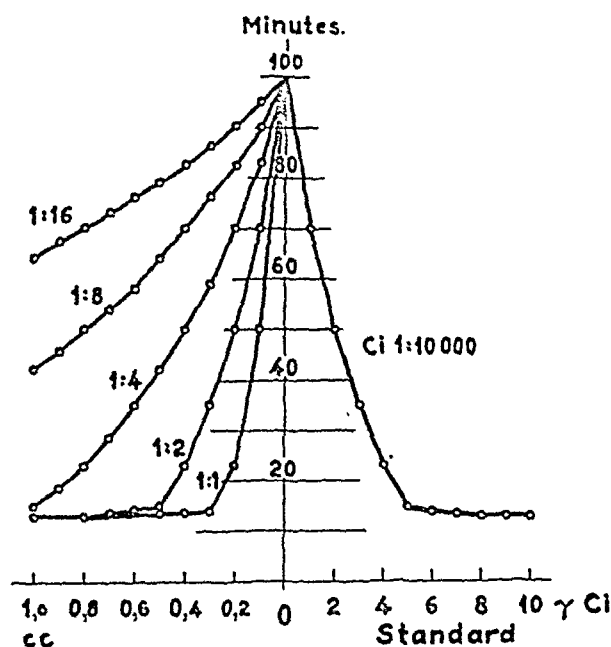


Fig. 6. Form of the citrate-curve for different dilutions of the fluid to be tested.

a "false level" high up. The risk of this error occurring becomes greater the less Ci the solution contains; i. e. the longer the decoloration-times become. With a long series of tubes it may play a minor part, but in routine work limited to three or four tubes the error may be considerable if attention is not directed to it. It is therefore safest to try to get a dilution of the test fluid that will give a curve as nearly as possible identical with the standard curve.

It is evidently inattention to the conditions pointed out here which has led to LENNÉR's (1934) statement that "the level is higher in estimations of fluids with low Ci-content", which was later confirmed by SJÖSTRÖM

(1937). It is then quite natural that he is able to stop the inhibitive action, upon which the high level is thought to depend, by adding Ci in even small amounts. No doubt the same misunderstanding is responsible for SjöSTRÖM's reference to "sera with a high level", although he publishes at the same time reports of an experiment on *the same* serum which in the 1:3 dilution had a much higher level, in the 1:2 dilution a considerably lower one, although the inhibition there ought to be greater. In the higher dilutions he could find no difference in decoloration-time for those serum-quantities, which in the lower dilution gave a difference of 19 minutes.

From Fig. 6 it is also to be seen that by the Thunberg method as customarily disposed (with 10 γ MB in each tube) it is possible with relatively high accuracy to estimate the Ci-content of solutions containing down to 5 γ of Ci per cc; for lower concentrations the values are more uncertain.

The Systematic Deviation of the Serum-Citrate Curve.

All who have occupied themselves with estimations of the citric acid in the serum have found that the serum-curve is often subject to a systematic deviation of the values, so that these fall with rising amounts of serum. The ascending leg of the curve is more horizontal and the level often somewhat higher than in the standard curve. It may even happen that the value for the 0.2 cc. tube is double as large as for the 0.6 cc. tube. Which of these values is the more correct? ÖSTBERG (1934) carried out tests with retarders and accelerators added to known quantities of Ci and in both cases found the least deviation from the right value in the lowest part of the ascending leg of the curve.

Two alternatives must be borne in mind: 1) That serum contains *retarding* substances whose action is the greatest in the tubes containing most serum, i. e. where their concentration is the greatest. That this can actually be the case is readily seen from the frequent failure of attempts to press the level of the X-curve down to that of the standard curve, even by reducing the dilution; instead, a longer decoloration-time may even be got the more serum that is loaded into the tubes. 2) That serum contains *accelerating* substances whose action is stronger the less Ci the tubes contain, that is to say, the larger the proportion of MB decoloration dealt with by the spontaneous activity. It seems very natural to suppose that the *most important cause of the systematic deviation is that*

serum contains, besides Ci, other substances that serve as donors for dehydrogenases in the enzyme solution. It has been previously shown that the spontaneous dehydrogenation declines in intensity in the course of the experiment as a result of the successive decrease in substrate concentration. When this becomes very low, an extremely small addition of donor substances suffices (when decoloration-time is relatively long) to cause a distinctly accelerated decoloration. This effect becomes the more pronounced, and the values consequently the higher, the further up the ascending leg of the curve they fall.

That these rising values actually depend on an intensified acceleration, and not on an intensified retardation, is evident from, e. g., the following experiment in which a series of tubes gave a particularly pronounced systematic deviation of the values (serum diluted 1:2):

Serum quantity ..	0.1	0.2	0.3	0.4	0.5	0.6 cc
Ci-value	37	25	21.7	19.5	17.6	15 γ /cc

The 0.2 cc serum was replaced by 0.1 cc serum + 0.1 cc Ci-solution containing 1 γ of Ci. If the value for the 0.1 tube, 37 γ , were correct, the tube with 0.1 cc serum + 0.1 cc Ci-solution ought to be found to contain $1.85 + 1 = 2.85 \gamma$, since this tube does not contain serum in a larger concentration than the 0.1 tube and consequently any retarding action cannot be so great either. Actually it proved to contain about 2.3 γ , and hence the acceleration induced by other donors was considerably less with this shorter decoloration-time.

It must also be remembered that the systematic deviation may also be due in part to the presence in the serum of co-enzyme, which, while not affecting the Ci dehydrogenation, presumably does affect the spontaneous. In favour of this argues the fact that the systematic deviation oftener becomes more pronounced with fresh serum than with serum that has been kept for a day.

There is thus good reason to expect that the Ci-curve for serum will be affected, in most cases, by an acceleration — greater or less — that predominates in the upper part of the curve and, in many cases, by a retardation that predominates in the lower part of the curve. Consequently, *the correctest values are most likely to be found just above the bend of the curve*, where these two factors neutralize each other. But this presupposes the use of a long series of tubes for the serum Ci estimations in order to ensure that the point at which the level-line bends round into the ascending leg and the proper values really can be determined. Or else an attempt must be made, after preliminary tests, to adjust the dilution so that

the serum-curve as nearly as possible resembles the standard curve, in which case only the three tubes which in the complete curve fall immediately above the bend need be included in the calculation. The latter alternative is distinctly more practical and accurate. But if, in such a case, the dilution happens to be excessive (so that even in the top tube the decoloration-time for the serum-curve is considerably higher than that for the standard curve), this will mean that three values are chosen high up on the complete curve, where the uncertainty is *per se* considerable and where the systematic deviation asserts itself most, and hence the value obtained will mostly be too high. That is why with *apparently the same technique* widely different values are obtained from the same serum, depending on the dilution selected.

HIRSCHLAFF-LINDGREN (1937) has estimated the Ci-content of horse serum, in which work she also made use of a control curve of serum solution with an addition of Ci-solution. She then comes to the conclusion that the method only detects 90 % of the serum Ci. But in her experiments an upper part of the curve is compared with a lower part, and as the systematic deviation of the values should suffice to explain the difference, her conclusion that the method only accounts for 90 % of the serum Ci is not justified on the basis of her experiments.

For routine estimations I proceed as follows. The standard curve is adjusted by strength of enzyme and intensity of illumination so that the level lies at about 15 minutes and the spontaneous decoloration at 80—100 minutes. So rapid a decoloration is more advantageous from all points of view, e. g. the estimation takes less time, the sources of error are fewer, multiplying appreciably with lengthening decoloration-time. The contact-point with 10 γ of MB then comes to lie between 5 and 6 γ of Ci. The enzyme solution can be adjusted to suitable quality by varying the dilution, the degree of stirring and the extraction-time. After preliminary tests with 0.4 cc. of serum solution against 0.4 cc. of standard solution, the serum is so diluted that the solution may be expected to contain about 10 γ per cc., and three tubes with respectively 0.5, 0.4 and 0.3 cc. are used for the estimation. If with a dilution of, say, 1:8, the serum gives a value between 70 and 90 γ per cc., there is reason for believing this correct. Should there be considerable deviations, however, it may be advisable on repeating the estimation to employ another dilution that gives better agree-

ment with the standard curve. As the method is so delicate that accidental factors may creep in, the same serum should as a general rule be estimated on two separate occasions.

Calculation of the C_i -values.

For the working out of the C_i -values I have used THUNBERG's graphic method, which is grounded on the basic principle that under equal conditions equal decoloration-times mean equal quantities of C_i in the tubes.

LENNÉR's method of calculation is more tedious and would seem to give somewhat more uncertain values owing to the difficulty of exactly determining the position of the contact-point and to the fact that the level of the serum-curve frequently lies somewhat higher than that of the standard curve. His statement that the contact-point values of the serum and standard curves are equal even if the serum-curve level is higher is theoretically inaccurate, although in most cases the assumption does not lead to great errors in practice. It is correct provided the C_i -activity and the spontaneous activity are subject to an inhibition of equal degree, so that the ratio between them remains unaltered. But this probably occurs only in the "artificial serum" with which LENNÉR has sought to prove the accuracy of his statement: in that serum there occurs only an inhibition by salts, no substances are there that increase the spontaneous activity, as there are in natural serum.

SJÖSTRÖM's method of computation is only a special case of THUNBERG's, applicable if the curve resembles a rectangular hyperbola: a mean value for the serum-curve is compared with a mean value for the standard curve, whereas in THUNBERG's method the value of each serum-tube is first computed and then the mean is taken. The advantage of SJÖSTRÖM's method is considered to be that it renders possible a calculation of serum-curves that lie so high above the standard curve that they cannot be evaluated direct. Curves of this type, however, give such unreliable results that they ought to be discarded from the outset. No real advantage would appear to be offered by SJÖSTRÖM's method as against THUNBERG's. SJÖSTRÖM has subjected LENNÉR's method to a severe criticism, based on a series of experiments showing that LENNÉR's method may lead to such absurd results as that a serum, after addition of C_i , shows a lower value than without this addition. But these experiments of SJÖSTRÖM's are faulty owing to his having used serum in so great a dilution that he has not been able to determine the real position of the level and the contact point, just those characteristics upon which LENNÉR's method is founded. In his series SJÖSTRÖM does not use so large quantities of serum that there is even any theoretic possibility of reaching the level-line. It is regrettable that this excessive criticism, based as it is on inexact experiments, should

have been instrumental in discrediting the enzymatic Ci-estimating method as such.

The accuracy of the Enzymic Method of determining Ci.

By THUNBERG's method it is possible to determine quantitatively the amounts of Ci added to serum or other biological fluids, as is evidenced by many experimental investigations (LENNÉR, SCHERSTÉN, SJÖSTRÖM) and as I can confirm on the strength of a number of experiments. There are, then, tolerably good grounds for concluding that even those values directly obtained by the method represent the actual Ci-content of the fluid concerned. A more direct check has been devised by SCHERSTÉN (1936). He made a series of estimations on genital-gland secretion simultaneously by THUNBERG's method and the purely chemical pentabromacetone method, and found no systematic difference between them.

Respecting the *single estimation mean error* the following figures are available: LENNÉR (1934), on the basis of the differences obtained at duplicate estimations of 19 sera, found the mean error to be 0.52 γ (which represents about 2.5 %). From 31 duplicate estimations SCHERSTÉN (1936) calculated the relative mean error of a single estimation at 2.57 ± 0.33 %; he expresses the difference in percentage of the mean value of the two estimations because equal relative errors are equally probable. SJÖSTRÖM (1937) computed a mean error that under ordinary conditions should not exceed ± 0.8 %. But this figure seems more to be an expression of how closely his curves approach a rectangular hyperbola, and it is debatable whether the figure is a measure of the accuracy of the method. In any case, his calculation only partly covers the sources of error attaching to the method. A reliable measure of the single estimation mean error can presumably only be obtained by carrying out two estimations on a sufficient number of sera on separate occasions and with different enzymes, so that all sources of error will have a chance of exercising their effect. From such a series of 28 duplicate estimations GRÖNVALL (1937) arrived at the value 3.3 ± 0.44 %.

On comparing some duplicate estimations I found that the dispersion could sometimes be still greater than is indicated by the figures adduced above. For that reason I have since made double estimations when possible. A comparison of all the 150 duplicate estimations made during the period $^{12}/_9$ 1939— $^{14}/_2$ 1940 gives the result submitted below: the dispersion is calculated according to the formula $\sigma = \sqrt{\frac{\sum D^2}{2n}}$, where n is the

number of duplicate estimations and D the difference expressed in % of the mean value of the two related determinations, since equal relative errors are equally probable.

Diff %	No. of Duplicate Estimations	pD^2
0	8	0
1	17	17
2	15	60
3	20	180
4	14	224
5	12	300
6	19	684
7	10	490
8	12	768
9	9	729
10	2	200
11	7	847
12	3	432
13	1	169
15	1	225
<hr/> n = 150		ΣD^2 5325
$\sigma = \sqrt{5325/300} = 4.21 \pm 0.24 \%$		

In routine work, therefore, the single estimation mean error is $4.21 \pm 0.24 \%$. Assuming the accuracy of the value obtained from a duplicate estimation to be $\sqrt{2}$ greater than that of a single estimation then the accuracy would be very near 3 %. Consequently, should two values, each determined by duplicate estimations, show a difference of 12.6 %, this difference is to be regarded as significant.

Another series of 200 duplicate estimations, most of which were carried out during the spring (the whole series ¹¹/₁—¹²/₆, 1939), when according to experiences the enzymic method is less accurate, gave by the same computation a slightly greater dispersion: $4.58 \pm 0.23 \%$.

Comparative Investigations with the Pentabromacetone Method.

The Thunberg method has certainly also been used for the investigation of the Ci in tissues (GEMMILL, 1934), but, as this can only be done after an extensive preliminary treatment, it is no doubt more convenient and exact to use a purely chemical method for this purpose.

A review of the various chemical methods for determining Ci has already been given by SCHERSTÉN (1936). The very sensitive colour reaction described by FÜRTH & HERRMANN (1935) — not included in Scherstén's review — has scarcely been elaborated yet for exact quantitative determination of Ci. In the present work I have used the pentabromacetone method as modified by PUCHER, SHERMAN and VICKERY (1936). It is founded on the fact that Ci is oxidized to pentabromacetone, which with sodium sulphide is transferred to a coloured substance, this being measured with the Pulfrich photometer and the Ci-values thereupon obtained from a calibration curve. By this method quantities between 0.1—1 mgm. can be determined to an accuracy of $\pm 5\%$. Its sensitiveness and accuracy are accordingly essentially lower than the Thunberg method's, and the dispositions described, which I have followed in detail, take up at least as much time.

Besides estimations of the Ci-concentration in tissues and whole blood I have made some experiments with the pentabromacetone method on serum for comparison with the Thunberg method. The values obtained, however, are not directly comparable, since the chemical reaction is given exclusively by Ci, and not by *cis*-aconitic acid and isocitric acid (BREUSCH, 1937), which are in some degree of equilibrium with Ci in the tissues and which are also included in estimations with the enzymic method. Statements as to the proportions in this equilibrium differ somewhat: at least 75 % Ci (BREUSCH, 1937); 90 % Ci, 10 % isocitric acid, and minimum concentration of aconitic acid (MARTIUS, 1938); 80 % Ci, 16 % isocitric acid, and 4 % aconitic acid (JOHNSON, 1939). No investigation is available as to the conditions in the serum, and, of course, nothing is known of the distribution in blood from different parts of the body. Large series of parallel estimations with the chemical and enzymic methods would be required to investigate this interesting problem and the question of the transformation of orally or parenterally administered Ci, since both the methods are burdened with an error percentage that is rather large in proportion to the differences which are likely to be found. In view of this I have not gone into the question here, my object being to ascertain whether biological experiments give the same result when Ci is estimated with the purely chemical method as when it is estimated with the enzymic.

In a series of 13 estimations on serum from arterial blood the pentabromacetone method gave values which were on an average 16.5 % ($16.5 \pm 2.9\%$) lower than those given by the Thunberg method.

The degree of accuracy I attained with the pentabromacetone method has not been determined on the strength of any large series. A comparison of nine duplicate estimations gives the *single estimation mean error, calculated as described above for Thunberg's method, as 9.56 ± 2.23 %*.

Summary.

The complex dehydrogenating process that underlies THUNBERG's enzymatic method of Ci estimation is discussed. My investigations have shown that:

The specific enzyme system in the cucumber-seed extract for Ci shows good stability, even at a temperature of 35° C. The spontaneous dehydrogenating activity in the enzyme solution, on the other hand, rapidly declines owing to exhaustion of donor substances. The cucumber-seed used contains itself about 0.035 % of Ci, which must be dehydrogenated before the typical citrate-curve can be got by adding Ci.

The specific enzyme system in the cucumber-seed extract for malic acid is less stable than that for Ci and for hexose-diphosphoric acid.

The activity of the Ci-specific enzyme system in the cucumber-seed varies but slightly, if at all, in response to changes in the hydrogen-ion concentration within the range pH 6.6—7.6, whereas the spontaneous dehydrogenation declines with falling pH and ceases almost altogether at pH 7. The dehydrogenation of malic acid and hexose-diphosphoric acid declines with falling pH.

The most important cause of the systematic deviation in the values of a serum citrate curve is doubtless that serum, in addition to Ci, contains quantities of substances which serve as substrates for dehydrogenases in the enzyme solution.

For the *practical* performance of the Ci estimation the following points have proved to be of importance:

The stirring of the cucumber-seed extract must always be of equal degree if equal enzyme strength is wanted in the extracts.

If the spontaneous dehydrogenation in the extract is too strong, a suitable enzyme solution is not obtained by diluting, but by keeping the solution in room temperature for a while.

The lighting should be kept constant, preferably by means of artificial lights in a room screened from daylight.

The dilution of the solution to be tested should be so selected

that the X-curve follows as closely as possible the course of the standard curve. The most accurate values lie immediately above the bend of the curve. In routine work the single estimation mean error proved to be 4.21 ± 0.24 %.

The pentabromacetone method in PUCHER, SHERMAN and VICKERY's modification, which is specific for Ci, generally gives lower values for serum than the Thunberg method, which also includes any preformed isocitric acid and *cis*-aconitic acid that may be present.

CHAPTER II.

The Citric Acid Metabolism in the normal Mammalian Body.

Review of previous investigations.

On the strength of his investigations into the effects of certain vegetable acids, among them citric acid (Ci), on the elementary respiration in minced frog muscle, THUNBERG (1911 a, b, c) surmised that Ci is a normal intermediate product in the metabolism of muscle. BATELLI & STERN (1911) found that the consumption of oxygen was also increased by Ci in the liver, kidney and muscle of mammals. The specific dehydrogenase for Ci was subsequently shown to be present in most tissues. WISHART (1923) demonstrated citricodehydrogenase in phosphate extract of liver, and BERNHEIM (1928) submitted a method of extracting Ci-dehydrogenase from liver, which method has since been improved by SCHERSTÉN (1936) and SJÖSTRÖM (1937). As a result the liver-cells have been shown to contain Ci-dehydrogenase in abundance. Using a modification of the pentabromacetone method, LANGECKER (1934) measured the breakdown of Ci in various minced tissues from the rabbit. Her experiments showed that the liver possesses great ability to destroy Ci, the kidney somewhat less, and muscle very little. Ci added to intestinal contents was destroyed in only a slight degree, and hence bacterial decomposition of Ci in the intestinal tube is not of any importance. Nor was Ci destroyed in the blood, a fact that had already been shown by SALANT & WISE (1916).

These authors were the first to use a tolerably reliable method for the study of the *in vivo* Ci metabolism of the mammalian body. With their modification of the DENIGÈS method they were unable to detect Ci direct in blood samples from rabbit, cat, and dog. But when they administered Ci orally or subcutaneously (in such large doses that the experiments were more of toxicological interest), a considerable amount of Ci was discovered in the blood. After intravenous injection the Ci vanished quickly from the blood. Only a small proportion was found in the urine, mostly after subcutaneous injection, in rabbit urine on an average 12 %, in cat 30 %, although the diuretic effect was stronger in rabbits.

The first quantitative investigation of the Ci excretion in man was carried out by ÖSTBERG (1931). The 24-hour amount of Ci varies normally

between 0.2 and 1 gram. Intake of Ci by mouth, even in so large a dose as 40 grams, does not appreciably increase the Ci excretion. Practically all the Ci-intake is therefore oxidized in the body. This result was soon confirmed by other investigators.

KUYPER & MATTILL (1933) studied the Ci-content of blood from different parts of the body in rabbits, but could not find definite evidence of where in the body the Ci was formed or oxidized; the renal-vein blood, however, contained less Ci than the arterial blood. Protracted inanition lowers the serum citric acid (C/s, given hereafter in γ = micrograms per 1 cc.), but even then Ci goes on being excreted, which indicates that it is endogenously formed. (It was earlier known that Ci is introduced with the food, since a large number of foods contain Ci; see ÖSTBERG (1931) for the literature on this subject.) BOOTHBY & ADAMS (1934) found the same in the case of the dog, and it was demonstrated still more convincingly by SHERMAN, MENDEL & SMITH (1936 a). They followed the Ci excretion in dogs during a long period of experimental alkalosis, when the 24-hour amount of Ci in the urine amounted to 100—300 mgm. against 1.3—8 mgm. normally. During three stated test-periods the Ci excretion was respectively 5, 10 and 7 grams larger than the Ci intake with the food, which clearly shows that Ci is a product of metabolism. For there is no store of preformed Ci in the tissues, the Ci-content there being of about the same order as that of whole blood, as PUCHER, SHERMAN & VICKERY (1936) have shown with their modification of the pentabromacetone method¹. In liver, however, they found very little Ci. (Still earlier GEMMILL (1934) had been able to show the presence of Ci in the tissues, while LANGECKER (1934) stated that normally the tissues do not contain Ci.) The dog excreted with the faeces normally 0.4—0.8 mgm per day, and this quantity was increased very slightly after oral administration of Ci. SHERMAN, MENDEL and SMITH (1936 b) studied the C/s in dogs after oral administration of Ci: after 1 gram of Ci per kg of body weight the C/s increased to 2—4 times the normal value, reaching maximum after $1\frac{1}{2}$ — $3\frac{1}{2}$ hours and returning to normal within $3\frac{1}{2}$ — $7\frac{1}{2}$ hours. Simultaneous estimations of Ci in blood and urine appeared to point to the occurrence of a renal threshold for Ci in the dog, though this threshold value varied widely in different individuals.

Still earlier, the C/s had been investigated after oral Ci administration in tolerance tests on man. THUNBERG (1933) found, in a normal subject, a moderate rise (38.4 % after 15 grams of Ci) that lasted some hours, and, in a patient with lesion of the liver parenchyma, a more powerful and more prolonged rise. Even 5—8 grams of Ci by mouth give a distinct elevation of the C/s, with maximum after 1—3 hours (LENNÉR, 1934). In his comprehensive and important work on the C/s in hepatic diseases SJÖSTRÖM (1937) reports that 1—2 grams of Ci by mouth scarcely raise the C/s in normal persons, while such a dose may produce a distinct and lengthy rise in patients with a lesioned liver parenchyma. Tolerance tests

¹ Cf. footnote p. 65.

with Ci given orally can therefore be a valuable supplementation of the liver-function test that estimation of the C/s constitutes. After intravenous injection of 0.5 gram of Ci the C/s conditions are substantially the same: in normal persons a moderate rise, usually with a return to the normal value in 30 minutes; in abnormal hepatic function with a C/s already high a powerful and protracted rise. GRÖNVALL (1937) followed the C/s in rabbits at relatively short intervals after slow intravenous injection of fairly large amounts of Ci: the transient rise was slight as against the Ci quantity supplied, and hence these tests, too, show that the body has great possibilities of coping with Ci introduced into it.

In connexion with his clinical investigation SJÖSTRÖM (1937) also made perfusion tests on isolated liver from rabbit, cat, and dog. He found that the liver has an enormous power of metabolizing Ci: even when the perfusion fluid contained 100 times the normal amount of Ci, this was restored to the normal value by a single passage of the perfusion fluid through the liver. Thus the liver acted as an efficient Ci filter, up to 20 grams of sodium citrate being removed with ease from the perfusion fluid by a rabbit liver. On perfusion of muscle preparation he obtained no corresponding consumption of Ci. In experimental lesion of the liver induced in rabbits with allyl formate, the C/s gradually rose, and on perfusion a liver lesioned in this way proved less able than normally to metabolize Ci.

The knowledge we possessed of the Ci metabolism of the body at the time the present work was commenced may be summarized as follows: Ci is taken into the body with the food but is also formed endogenously. The general occurrence of Ci in all body fluids suggests that this formation takes place fairly generally. The body possesses a very great power to break rapidly down intaken Ci, only a very small proportion being excreted in the urine and faeces. This breakdown of Ci is mainly effected in the liver, which has proved to contain an abundance of Ci-dehydrogenase and which at perfusion tests has shown an enormous Ci-metabolizing capacity.

The Citric-Acid Content of the Serum after Intravenous Administration of Citrate.

My own experiments started with some Ci tolerance tests on rabbits and cats. By following the C/s at sufficiently short intervals after a one-time intravenous dose of Ci I was able to get a rough idea of the extent of the Ci breakdown and of a suitable dose that does not elevate the C/s too much above the normal concentration. *Fig. 7* with text embodies some typical

tolerance tests, which have been confirmed by many similar ones.

For the experiments I used the preparation *Sodium citrate Analar*, British Drug Houses. The dosage is given in corresponding amounts of citric acid (with 1 mol. H_2O), since as standard both for the Thunberg and pentabromacetone methods I used *Acidum citricum cryst. pro analysi Schering-Kahlbaum*, which contains 1 mol. H_2O (which is possibly intra-

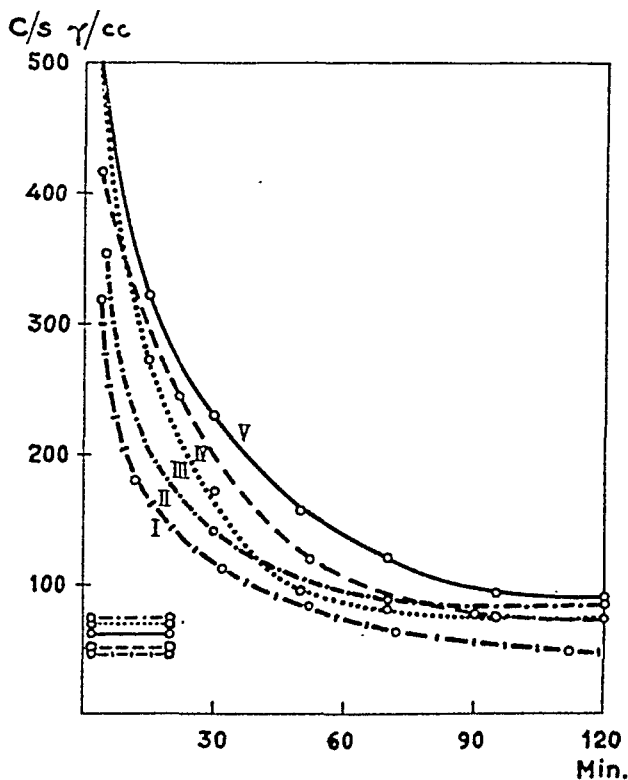


Fig. 7. *C/s after intravenous administration of Ci.*

- I. Cat, 3.5 kg. Pernocton 0.7 cc/kg. 43 mg *Ci*/kg.
- II. Rabbit, 2.1 kg. Urethane 1.25 gm/kg. 60 mg *Ci*/kg.
- III. Cat, 2.4 kg. Urethane 1.25 gm/kg. 100 mg *Ci*/kg.
- IV. Rabbit, 2.5 kg. Not narcotized. 86 mg *Ci*/kg.
- V. Rabbit, 3.9 kg. Urethane 1.25 gm/kg. 100 mg *Ci*/kg.

molecularly bound, THUNBERG 1933). By intravenous administration one avoids absorption inequalities and can reckon with an exact quantity. The speed of injection, which is entirely decisive of the toxicity of intravenously introduced *Ci*, was kept between 1—2 cc. per minute (according to size of animal) of the isotonic 3 % solution: in doses up to the highest used here, 100 mgm. per kg., it does not produce any pronounced reaction. The pharmacological effects of *Ci* are not dealt with in this work, and

were not recorded. The blood samples for Ci-estimation (3—4 cc.) were drawn from the A. femoralis or carotis of the narcotized animals, from the auricular vein of non-narcotized rabbits. As narcotic I used in most cases urethane in relatively small dose, 1—1.25 gm. per kg., subcutaneously, re-inforced with ether for operations, in some cases pernocton.

The experiments confirm earlier observations of the body's great power to break down Ci. This makes it possible to study the metabolism in fairly short experiments, which very much facilitates the experimental analysis. The curves show that *Ci disappears from the blood the more rapidly the higher the concentration is.* This rapid fall at high concentration cannot be due solely to a possible levelling-out of the concentration in the tissues just after the injection, for it is also found at pauses in long continuous infusion, when any such levelling-out ought already to have occurred. In one such experiment on a dog the C/s fell in 90 seconds from 925 to 700 γ per cc, in another from 685 to 565 γ per cc. In an experiment on a rabbit, in which the C/s had been forced up to 1540 γ per cc., it fell by over 400 γ in 70 seconds.

A significant point is that *the breakdown of Ci in the narcotized animals seems to proceed at the same rate as in the non-narcotized*, which suggests with a rather high degree of probability that other results gained from experiments on narcotized animals are also valid for the normal Ci metabolism.

SALANT & WISÉ (1916) found, as was mentioned before, that the urinary Ci excretion after subcutaneous administration was larger in the cat than in the rabbit, and from this they concluded that the rate of oxidation of Ci is considerably quicker in the rabbit, as being a herbivore. This logical conclusion received no confirmation on actual measurement in my experiments. On the contrary, the curves show that Ci is eliminated more rapidly in the cat, and therefore the greater Ci excretion cannot be due to the C/s remaining high for any considerable length of time.

The Citric-Acid Content of the Red Blood Corpuscles.

In order to compute the Ci metabolism in certain perfusion experiments it is necessary to have some conception of the Ci-content of the red blood cells, since the Ci estimations are carried out on serum or plasma but the fluid volumes have reference to blood. Some experiments relating to this question are therefore inserted here.

NORDBÜ and SCHERSTÉN (1931), using the pentabromacetone method, estimated the Ci-content of the red blood corpuscles in a case in which the plasma contained 35 γ per cc. They found about 10 γ per cc., a part of which, however, must be referred to plasma left on the cells. PUCHER, SHERMAN & VICKERY (1936) determined the concentration in the total blood and plasma and from that reckoned the amount in the corpuscles. They found considerably higher values: the plasma/corpuscle quotient was about 1.6.

I have made some indirect determinations by the Thunberg method, adding a weighed quantity of Ci to 100 or 200 cc. of heparinated blood, which was then allowed to stand at least 12 hours. The C/s was thereupon determined. The corpuscular volume had been determined with a haematocrit and the C/s had also been estimated before addition of Ci. With these data I could fix how much of the added Ci was present in the plasma, and from this estimate the amount in the corpuscles. Later on, some experiments with the pentabromacetone method were made to determine the Ci-content of whole blood and plasma, and the amount in the red corpuscles was calculated from this. The results are given in the following tables.

Thunberg Method:

Plasma Ci γ /cc	Bl. Corpuscles Ci γ /cc	Plasma Vol %	Plasma/Cells quotient	Ci in Blood in % of C/s
Rabbit 278	70.0	62.5	4.0	72
» 305	60.8	57.0	5.0	65
» 248	60.0	74.5	4.1	81
» 313	37.0	59.0	8.5	64
Dog 270	70.0	65.0	3.9	74
» 185	22.0	48.0	8.4	54

Pentabromacetone Method:

Plasma Ci γ /cc	Total Blood Ci γ /cc	Plasma Vol %	Plasma/Cells quotient	Ci in Blood in % of C/s
Dog 57.6	33.0	52.0	9.0	57
» 34.5	17.3	43.0	8.0	50
Cat 428.0	325.0	64.0	3.0	76
» 43.5	32.6	62.0	3.0	75
Rabbit 169.0	95.0	52.0	11.4	56
» 1140	776	62.5	6.7	68

Of neither procedure can any great accuracy be expected. The values, in fact, show rather wide deviations, but in no case did I find such high values as PUCHER and others. *The concentration in the corpuscles is probably five to six times lower than in the plasma.*

When measuring the Ci metabolism I took as an approximate value for the ratio of whole blood to serum the percentage obtained if the serum-volume percentage is increased by about $\frac{1}{10}$. For instance, if according to the haematocrit reading the serum volume is 65 %, the Ci-content of whole blood is assumed to be $65 + 6.5 =$ about 72 % of C/s. This value probably comes rather near the correct one (see the tables).

The Citric Acid Metabolism in Perfused Isolated Liver.

Even if the breakdown of Ci after intravenous administration is very rapid, it still takes at least one hour for the C/s to fall to its initial value. This is longer than would be expected if the liver immediately oxidizes the Ci in the blood flowing through it, as SJÖSTRÖM (1937) found in his perfusion experiments. I therefore repeated these experiments on rabbits and cats.

I used the perfusion apparatus and followed the preparation technique employed at the Lundsgaard Institute (LUNDGAARD, NIELSEN and ØRSKOV, 1936), the foremost advantage of which is that the circulation through the liver need not be interrupted more than at most two minutes. The method was last described by BLIXENKRONE-MØLLER (1938), with whom I have had an opportunity of studying the technique. As haemolysis must be avoided where enzymic Ci estimations are concerned, I could not defibrinate the blood, but used heparinated blood, when possible undiluted in order to approximate physiological conditions as closely as possible. The blood was fully oxygenated and its reaction stabilized by bubbling $O_2 + 5\% CO_2$ through it. A period of 15–20 minutes was allowed to balance and stabilize the conditions before the experiment proper was commenced. The latter was not unduly prolonged, even if technical considerations permitted this, for a tolerably normal metabolism can scarcely be expected to last more than at most a couple of hours. Ci was either added as a one-time bulk dose (then at such speed in relation to the flow volume per minute that it could be expected to spread uniformly over the entire volume of blood) or was infused continuously over a long period. The volume of blood was measured at the end of the experiment and the Ci-content was calculated with the aid of the haematocrit value as already described. This calculation is admittedly approximate so far as absolute values are concerned. What is of importance, however, is that

conclusions can be drawn from the *relative* values as to the metabolism under different experimental conditions, if the calculations for the different periods of the experiment are carried out along the same lines.

The curves in *Fig. 8* show the Ci-content of the perfusion blood after addition of a one-time dose of Ci. I got curves agreeing with these from some ten other experiments in different modifications, even on perfusion with Tyrode's solution

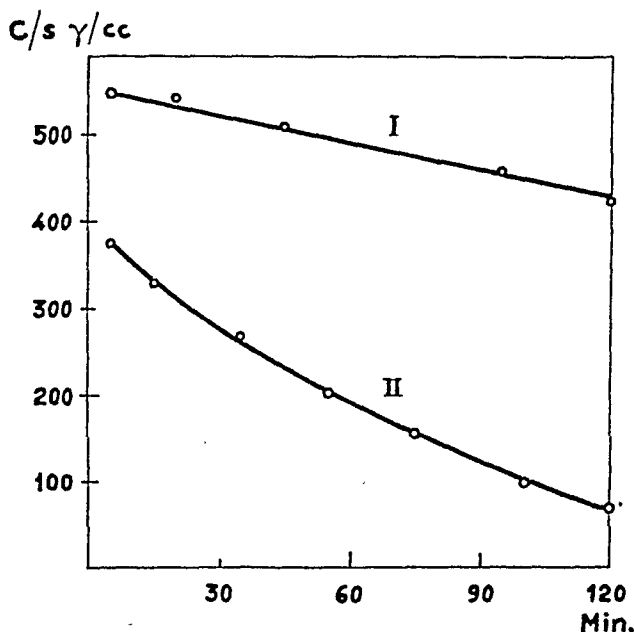


Fig. 8. The Ci-metabolism in perfused liver.

- I. Rabbit, 3.7 kg. Urethane 1 gm/kg. Wt. of liver 128 gm. Perfusion fluid 220 cc heparinated blood (with abt. 20 % Tyrode's sol.). Addition 90 mg Ci.
- II. Cat, 3.2 kg. Urethane 1 gm/kg. Wt. of liver 52 gm. Perfusion fluid 170 cc heparinated blood (with abt. 40 % Tyrode's sol.). Addition 60 mg Ci.

alone. The experiments show that the liver is by no means able to effect on immediate elimination of Ci from the blood; on the contrary, *the isolated liver has little power to metabolize Ci*. In the rabbit's liver the Ci metabolization is very low, in the cat's liver somewhat higher. But here, too, it is not sufficient to explain the body's power to break down Ci, even under the assumption that the metabolic processes are more active in the *in situ* organ than in the isolated. (As regards the consumption of O₂ there is a decline of this in the perfused

liver, by 25—35 % during a 2-hour period, BLIXENKRONE-MØLLER, 1938).

In the following experiment with continuous administration of Ci, in which the C/s did not rise noticeably above the level normal for rabbits, the consumption of Ci can be estimated at 1.3 γ per minute per gram liver.

Experiment 18. 11. 39. Rabbit, 3.4 kg. Urethane 1 gram per kg. Wt. of liver 120 grams. Perfusion fluid: 190 cc. heparinated blood with about 30 % Tyrode's solution; haematocrit value 27 %. Min. vol. 48 cc. Pressure 12 cm. H₂O. Temp. 37.8°. From 11.55 a. m. to 12.30 p. m. addition in uniform drip of 10 cc. 0.23 % sodium citrate in Tyrode's sol. = 20 mgm. Ci. C/s in perfusion blood (sample from stock tube): 11.55 a. m. 54.4 γ , 12.15 p. m. 90.2 γ , 12.35 p. m. 133 γ , all per cc., thus a rise of 79 γ per cc. during the period of 40 minutes. The increase of Ci in the liver and the blood was calculated as follows: To balance the Ci in the liver and in the blood left there, 30 cc., corresponding to one-fourth of the weight of liver, were added to the blood volume, which was 190 cc. The Ci-content of the blood was estimated at 80 % of the serum content, i. e. the increase was reckoned as 80 % of 79 γ , which is 13.9 mgm. on the total volume of 220 cc. Subtracting this from the added 20 mgm., we get a Ci consumption of 6.1 mgm., i. e. 1.3 γ per minute per gram liver.

For such an immense breakdown of Ci as SJÖSTRÖM obtained in his experiments I have been unable to find a definite explanation. His experimental method, however, has certain weaknesses, more especially the absence of effective arrangements for maintaining a constant temperature. The explanation may lie in this, especially as SJÖSTRÖM points out that normal liver temperature is an imperative condition for obtaining this great conversion of Ci. Furthermore, SJÖSTRÖM assumes that the liver consumption of O₂ is very moderate, and that a supply of oxygen from the portal blood cannot be expected. The fact is, however, that the consumption of O₂ by the liver tissue is very large; in the cat it is normally about 2.5 cc. per min., i. e. about ten times larger than in a corresponding amount of muscle (BLIXENKRONE-MØLLER, 1937). According to MC MICHAEL (1937) the liver of the cat gets about two-thirds of its oxygen from the portal vein, while the liver of the rabbit draws almost all its oxygen from the hepatic artery. The cat's liver can take up all oxygen in the blood flowing through it, but in the rabbit the O₂-content of the hepatic-vein blood never falls to such low values. Now, since the circulation through the hepatic artery is not maintained during the perfusion, the larger must be the O₂-content of the blood delivered by the portal vein. It is evident that the liver used in SJÖSTRÖM's experiments was in some way injured, so that the Ci and the Ci-dehydrogenase so abundantly present in the liver had come into contact in a way that does not occur under physiological conditions. On one occasion I obtained a considerable breakdown of Ci on

perfusion of a rabbit's liver, but in that case, in the course of the experiment, the stomach contents had digested the stomach wall and lower surface of the liver over a large area.

As control, with the liver *in situ*, I made an injection of Ci in a branch of the portal vein. This produced a vigorous rise of the C/s even in the arterial blood.

Of four concordant tests I am submitting the following: 10. 1. 38. Cat, 3.3 kg. Urethane narcosis. Ci slowly injected into the splenic vein in a dose of 32 mgm. per kg. Sample from carotid artery.

	Before inj.	7	20	40	60 min. after inj.
C/s	45.3	187	111	80.5	66.9 γ per cc.

Blood specimens drawn at the same time from the portal and hepatic veins did not, either, show so great a difference in the Ci-content as to suggest any considerable Ci breakdown in the liver. It must be observed that the hepatic vein also takes up blood from the hepatic artery, which always has (as is shown later) a lower Ci-content than portal blood. This agrees with a couple of experiments on rabbits by KUYPER & MATTILL (1933).

The samples used in my experiments were drawn in the following manner: The portal vein was laid bare, as was also the V. cava inf. in the thorax, which was opened under artificial respiration. The V. cava inf. was ligated immediately below the liver, whereupon the samples were withdrawn in rapid succession by puncture of the V. porta and of the thoracic part of the V. cava inf. The C/s of the hepatic-vein blood was in all cases lower and the differences, expressed in percentages of the portal value, were, for four rabbits, 7.8 %, 2 %, 9.4 % and 7.1 %, for a cat, 2.6 %.

After this it was easy to see that so vigorous a Ci metabolism in the liver as that found by SJÖSTRÖM was already excluded by the fact established by several authors that the C/s can be raised by oral administration of moderate doses of Ci, this even in normal persons or experimental animals without any Eck-fistula mechanism.

The Citric Acid Absorption from the Intestine.

Since a certain amount of Ci is ingested in the food, it is but natural that one should find a higher Ci concentration in the portal than in the arterial blood. After oral intake of Ci the C/s in man and dog reaches, according to works previously cited, a maximum within $1\frac{1}{2}$ —3 $\frac{1}{2}$ hours and returns to the nor-

mal value in a few hours. The same seems to apply to the rabbit according to the following experiment:

Non-narcotized rabbits were given sodium-citrate solution through a stomach-sound, rabbit I 100 mgm. Ci per kg., rabbit II 300 mgm. Ci per kg. Ci estimated in blood samples from auricular vein:

	Initial Value	$\frac{1}{2}$	2	4 hours after Ci-administration
Rabbit I	51.0	70.5	57.0	52.0 % per cc.
" II	54.2	81.5	71.8	54.5 % " "

In two experiments the C/s was followed in the portal blood after injection of sodium citrate into the duodenum. The blood specimens were taken from a T-cannula inserted in the sup. mesenteric vein. In a rabbit, which had received 100 mgm. Ci per kg., the C/s rose in 45 minutes from its initial value of 102 % to 157 % per cc.; in a cat, which had been given 60 mgm. Ci per kg., it rose from 50.2 % to 79.2 % per cc. in 30 minutes.

Thus the absorption begins very rapidly, and according to previous tests it comes to an end in a few hours. Therefore it is natural that the C/s should fall during a fasting period. LARSHOLM (1934) found in rabbits on mixed rations a fall from about 100 % to 65 % after 24-hours fasting. I have followed the C/s in a couple of cases during a 4-day period, during which the rabbits were given only water.

	Normal Value	1	2	3	4 days inanition
Rabbit I	105	88.5	78.8	61.1	65.0 % per cc.
" II	78.5	63.5	63.0	55.0	51.4 % " "

The decline of the C/s appears to be most rapid during the first 24 hours, though it also continues afterwards. This fall, however, need not only be due to interrupted supply of Ci, but may be caused indirectly by onset of acidosis due to starvation. In practically all experiments in this work the animals had fasted at least 24 hours before being used. Then the spontaneous fall of the C/s takes place so slowly that it cannot possibly influence the result of the, generally, short experiments.

A higher concentration of Ci is however also found in the portal blood of experimental animals that have fasted up to 72 hours (see table below). In view of the very ready absorbability of Ci shown in tolerance tests, it is scarcely credible that any of the Ci that is preformed in the food can be left in the intestine all this time. Rather must it be supposed that this Ci is a product of bacterial processes in the intestine or of the metabolic processes taking place in the intestinal mucosa

during absorption of the food. *Thus it would seem that even under fasting conditions the intestine is continuously delivering Ci to the blood flowing through it.*

Animal	Starvation Days	Arterial Blood C/s γ /cc	Portal Blood C/s γ /cc	Diff. % of arterial C/s
Rabbit	3	42.7	53.6	25.5
»	3	42.5	60.5	42.4
»	3	66.6	80.2	20.4
»	3	62.0	79.4	28.1
»	3	57.4 ¹⁾	101.0 ¹⁾	75.6
Cat	2	61.0	79.9	17.9
»	1	53.3	72.7	36.4
»	1	67.8	80.3	18.4
»	1	57.8	72.9	26.1

¹ By the pentabromacetone method.

In the rabbit the large part of the coecum does not evacuate its contents after several days' starvation. That a *bacterial production of Ci* can play some part there is shown by the following experiments.

The contents of the coecum of a rabbit just killed were withdrawn, mixed, and divided into two portions. To one of these trichloroacetic acid was immediately added, while the other was allowed to stand for two hours at 39° before addition of trichloroacetic acid. The Ci-content was then determined by the pentabromacetone method. Of two rabbits which had fasted three days, one showed a concentration of Ci in the intestinal contents before and after incubation of 30 and 53 γ per gram, the other 49 and 64 γ per gram. In a third rabbit, which had been starved 36 hours, the Ci-content rose from 54 to 79 γ per gram. In this case the contents left in the stomach were also analysed and found to contain only 13 γ Ci per gram.

Citric Acid Content of Serum after Functional Elimination of the Liver and Portal Area.

In view of the rather inconsiderable oxidation of Ci that takes place in the liver, it may be expected that the Ci metabolism of the body will not undergo any notable change if the liver is put out of function. This was investigated by the following simple means.

The arterial branches to the portal region (coeliac art., sup. and inf. mesenteric) were first tied and after that the portal vein. Then the gastrointestinal canal could be removed after double ligation of the oesophagus and rectum. The liver was allowed to remain, as it cannot be totally removed without injuring the V. cava inf. It was accordingly still in

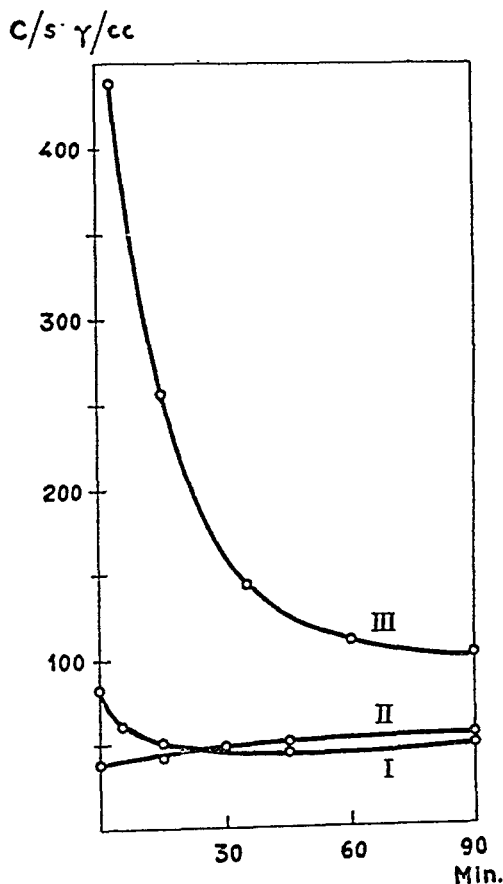


Fig. 9. C/s after functionally cutting off liver and portal area.

- I. Rabbit, 3.1 kg. Urethane 1.5 gm/kg. Ligation of arteries to portal area and of portal vein.
- II. Cat, 3.7 kg. Pernocton 0.7 cc/kg. Same operation.
- III. Rabbit, 4 kg. Urethane 1 gm/kg. Same operation. Intrav. injection of 60 mg Ci/kg.

"passive" communication with the circulation, but the very small inflow and outflow of blood via the hepatic veins arising at respiratory movements plays no part for the purpose of these experiments. Hence the gastrointestinal canal, pancreas and spleen were cut off from the circulation at the same time as the liver. The animals died in a few hours, even though

the decrease in the blood sugar resulting from the cutting-off of the liver (MANN and MAGATH, 1924) was counteracted by intravenous injection of glucose. For the rabbit this requires, according to SVEDBERG, MADDOCK and DRURY (1938), about 150 mgm. per kg. per hour, and in itself this amount has no effect on the C/s. On the other hand, after a rather large one-time dose of glucose (about 0.7 gram per kg. b. w.) I have found a distinct fall of the C/s in a couple of cases.

Three characteristic cases from these experiments are shown in *Fig. 9*. It will be seen that after functional elimination of the liver and portal region the C/s of the rabbit rapidly falls and subsequently remains at a low level. This is evidently due to the fact that the flow of Ci (prevented here) from the portal area is much greater than the liver's power of breaking down Ci. In the cat these two quantities are evidently better balanced, the C/s altering but little. Intravenously administered Ci is eliminated from the blood of the rabbit at about the same rate as in an intact animal, at all events to begin with. This clearly, shows that *the liver is not necessary for the breaking down of Ci in the body*.

As regards the method it may be objected that these "acute" experiments involve such shock effects from the operation that no conclusions can be drawn respecting the metabolism. But the experimental conditions are kept constant to some extent by the state of narcosis under which the animals lie during the entire experiment, before, during and after the operation. Then, too, in experiments involving operation in several stages, e. g. according to DRURY (1929) or the still more sparing one according to HIMSWORTH (1938), the pure effect of bye-passing the liver does not appear, as the animals have had ample time to adapt themselves to such changes in hepatic function as are implied in the diversion of the portal blood direct into the general circulation.

The Citric Acid Metabolism in an Eviscerated Preparation and in Perfused Muscle.

Previous experiments have shown that Ci can be oxidized in a preparation in which the kidneys and muscle play the greatest metabolic part. If, in addition, the renal vessels are tied, giving a preparation of predominantly muscle, there is a gradual but considerable rise in the C/s of both rabbits and cats (see *Fig. 10: I and II*), which cannot be interpreted otherwise than that *Ci is formed in the muscles*¹. No undoubted Ci-oxidation follows an intravenous supply of Ci to an eviscerated

¹ Cf. footnote p. 65.

preparation (Fig. 10: III, IV); the C/s certainly falls at first, but no doubt this only depends on a levelling-out of the concentration over the whole preparation, after which it remains stationary at high concentration only to rise again afterwards.

In such preparations the experimental conditions are not quite constant for any considerable time, since blood pressure and respiration are influenced rather early. I have therefore conducted some *perfusion experiments on isolated hindlegs of*

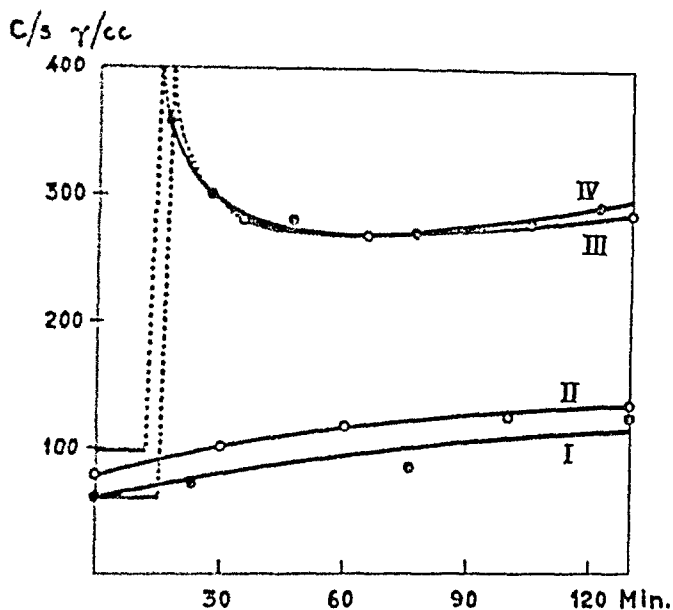


Fig. 10. C/s after abdominal evisceration.

- I. Cat, 1.7 kg. Pernocton 0.7 cc/kg. Continuous injection of adrenaline, total 0.1 mg.
- II. Rabbit, 3 kg. Urethane 1 gm/kg. Continuous injection of glucose 0.4 gm per hr.
- III. Cat, 3.8 kg. Pernocton 0.8 cc/kg. Adrenaline 0.1 mg. Intrav. injection of 28 mg Ctl/kg.
- IV. Rabbit, 3.2 kg. Pernocton 1 cc/kg. Adrenaline 0.1 mg. Intrav. injection of 27 mg Ctl/kg.

rabbits and cats, in which experiments the oxygenation and flow volume of the blood can be kept more constant and effects from other organs (lung, heart, brain) avoided.

For perfusion of the hind-legs I used the same apparatus as for liver perfusion, but kept the circuit closed in order to ensure sufficient pressure. I had access to a Dale-Schuster pump, by which the flow volume per minute, and with it the pressure, can be readily adjusted during the ex-

periment without interruption of the circulation. The lateral arterial branches were carefully ligatured, as were also all the vessels to the bladder and genitalia, and the entire posteriors were constricted at the beginning of the perfusion by means of a steel wire with a screw device with which the vertebral column could also be compressed. Otherwise the preparation leaks up into the anterior part of the body.

Of the experiments, the following may be submitted: 26. 2. 40. Rabbit, 4.35 kg. Urethane 1 gm. per kg. Perfusion fluid: 240 cc. heparinated blood with about 20 % Tyrode's sol.; haematocrit value 29 %. Min vol. 56—60 cc. Pressure 60—65 mm. Hg. Pulsation 70 p. min. Temp. 37.6°. The C/s of the perfusion fluid rose from 90.9 γ to 103 γ per cc. during a 50 minutes' period. After addition of 45 mgm. Ci the C/s sank from 348 γ immediately after the addition to 308 γ per cc. after 20 min. and 282 γ after 50 min.

In a similar experiment on a rabbit the C/s rose in 80 minutes from 73.1 γ to 105 γ per cc., and in one on a cat it rose in 45 minutes from 41.7 γ to 55.3 γ . After adding Ci to another hindleg preparation of a rabbit the C/s was as follows:

	5	15	30	50 min. after Ci addition
C/s	524	416	374	397 γ /cc.

That the rises were actually produced by Ci, and not by, e. g., hexose-diphosphoric acid, formation of which in the muscle was conceivable under these conditions (DEUTICKE & HOLLMANN (1939), was verified in one of the above-cited cases by the pentabromacetone method: the C/s rose from 67.2 γ to 84 γ . Nor did the curves for the Ci estimation according to THUNBERG deviate from the usual type, and the "level" did not reach lower than that of the standard curve.

The perfusion experiments, therefore, fully confirm the results obtained from eviscerated preparations: the Ci-content of the perfusion fluid increases during the experiment, and on adding Ci up to a high concentration no undoubted oxidation of Ci in the muscle is found.

Now this increase could depend upon the Ci-formation in the muscle here being brought about, or being accelerated, under the influence of other metabolic products that accumulate in a muscle preparation. *Ci is also formed, however, in muscle with normal metabolism.* In one series of experiments I took samples at the same time from the arterial blood and the peripheral part of the femoral vein of an otherwise intact animal. The results are furnished in the table given below and show that the C/s of the blood flowing out of the muscle is higher than that of the inflowing blood.

The animals were narcotized as usual. The venous blood was drawn by puncturing the femoral vein immediately after the latter had been

centrally clamped. The arterial blood was taken from the femoral artery on the other side, or from the carotid. (The difference is stated in percentages of C/s of the arterial blood.)

Animal	Art.	V. fem.	Diff. %	Animal	Art.	V. fem.	Diff. %
Cat	40.7	53.0	30.2	Rabbit	109.0	113.0	3.6
»	38.2	46.3	21.2	»	71.4	77.7	8.8
»	57.8	66.2	14.5	»	42.7	45.0	5.4
»	55.0	63.9	16.2	»	42.5	43.5	2.4
Dog	53.2	58.3	9.6	»	66.6	70.4	5.7
Rabbit	77.3	80.8	4.5	»	62.0	64.1	3.4

In rabbits the difference is distinctly smaller than in cats, but a higher Ci concentration is nevertheless always found in the venous blood. Substantial quantities of Ci, however, can be brought along with the copious blood flow from the total musculature of the body and yet the difference in Ci-content between arterial and venous blood may be small.

In *heart muscle* the behaviour of the Ci metabolism does not appear to be materially different from what it is in ordinary muscle. A perfusion test on a rabbit's heart failed to reveal any change in the Ci-content of the perfusion blood during the experiment.

The heart, which weighed 9.4 grams, was perfused from the aorta, in the usual Langendorff preparation, with 160 cc. of undiluted heparinated blood. The pressure was kept at 105 mm. Hg, which gave a blood flow of 29—26 cc. per minute; the heart beat well the whole time. For a period of 40 minutes the C/s remained unchanged, initial value 39.1 γ per cc., final 39.2 γ . After addition of Ci the C/s was at first 282 γ per cc., after 40 minutes 274 γ . The circulating quantity of blood was certainly large proportionately to the weight of the heart, for which reason small changes did not reveal themselves, but the blood can none the less be assumed to have passed through the heart muscle six or seven times during each period of the experiment.

The Citric-Acid Excretion in the urine.

The experiments hitherto described point to the kidneys as being the organ by which Ci is eliminated from the body.

According to the literature reviewed earlier, it was known that at most two per cent. of the amount of Ci supplied by mouth is excreted in the urine. After parenteral administration the urinary Ci excretion may be

considerably larger: SALANT & WISE (1916) had found 30 % in experiments on the cat, ORTEN & SMITH (1937) up to 40 % in the dog after such large intravenous doses as 400 mgm. of sodium citrate per kg. b. w. The excretion is accordingly bound up, as might be expected, with the actual Ci concentration in the blood: This is very vigorously augmented after

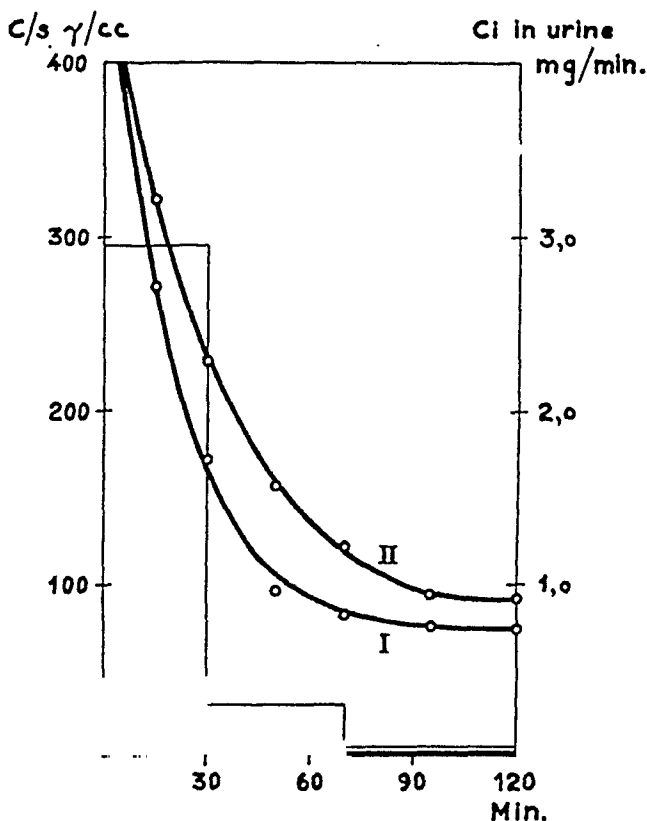


Fig. 11. C/s and urinary Ci excretion after intravenous injection of Ci.

- I. Cat, 2.4 kg. Urethane 1.25 gm/kg. Intrav. injection of 100 mg Ci/kg. Ci-concentration in urine (white field): before Ci-injection 69 γ, in first portion 18450 γ, in second 5890 γ, and in third 1480 γ/cc. Total Ci-excretion 103 mg or 43 % of the amount administered.
- II. Rabbit, 3.9 kg. Urethane 1.25 gm/kg. Intrav. injection of 100 mg Ci/kg. Ci-concentration in urine (black field): before inj. 188 γ, in first portion 11520 γ, in second 2520 γ, in third 1530 γ/cc. Total Ci-excretion 19 mg or 4.9 % of the amount administered.

intravenous injection of Ci, whereas after oral administration it does not attain such a height that the renal threshold is materially exceeded.

The most important precaution in a tolerance test designed to investigate the Ci excretion is to follow the C/s and to collect the urine at sufficiently short intervals, as the Ci elimination

is so transitory. Two typical experiments of this kind on rabbit and cat are described in *Fig. 11* and its accompanying text. These show that *the kidneys possess an exceedingly high power to concentrate Ci in the urine*. The most extreme case was in a dog, in which continuous infusion of Ci gave a urinary concentration of not less than 3.8 %. *This excessive rise of the Ci concentration in the urine presupposes a powerful elevation of the C/s, and it vanishes as rapidly as the C/s sinks*. Hence even a powerful increase in the concentration need not at all become visible if the Ci estimation is made on, say, samples of daily amounts; SALANT & WISE found no Ci in the urine after intravenous injection of 100 mgm per kg., just the dose used in the experiments recorded here. The experiments confirm the old observation that in cats the Ci excretion is considerably larger than in rabbits, 43 % and 4.9 % respectively of the same Ci dose. In two other cases the excretion in the cat was 27 % and 30 %, in four cases among rabbits 3.8 %, 8.2 %, 4.1 % and 4.8 %. This divergence must be due to a primary difference in the kidneys themselves, since the C/s keeps high for a longer time in the rabbit and might accordingly be expected to cause a larger excretion.

In the experiments the urine was collected through a cannula tied into the top of the bladder. By this simple means urine could be continuously collected even from rabbits, on which animals several earlier experiments had failed. Manipulations of the urinary organs are apt to give rise to oliguria or complete anuria through ureteral spasm or other causes, and this doubtless reacts on the passage of blood in the kidneys, since injected Ci is then not eliminated from the blood in a normal manner.

In an experiment on man one gram of sodium citrate was intravenously injected during four minutes, and the C/s rose from the normal 25 γ per cc. to 116 γ per cc. The collected urine from the first 45 minutes showed a Ci concentration of 1600 γ per cc., a moderate rise.

The Citric Acid Metabolism in Perfused Kidney.

If the kidneys are to answer for the greater part of the body's removal of administered Ci, it must be assumed that, collateral with the urinary Ci excretion, a considerable oxidation of Ci goes on in these organs. To investigate this aspect more closely I made a series of perfusions on isolated cat kidneys with heparinated blood. With this object the perfusion can be undertaken direct, without having a lung preparation, which other-

wise serves to "detoxicate" the blood of vasoconstricting substances.

The kidneys were left *in situ*. The aorta was clamped below the renal arteries and the inflow cannula was attached there. The outflow cannula was inserted in the V. cava inf. below the renal veins, whereupon the former was ligatured above the latter, and the cat was allowed to bleed through the renal vessels. Perfusion was then started, and simultaneously the aorta was ligated above the renal arteries, by which means the

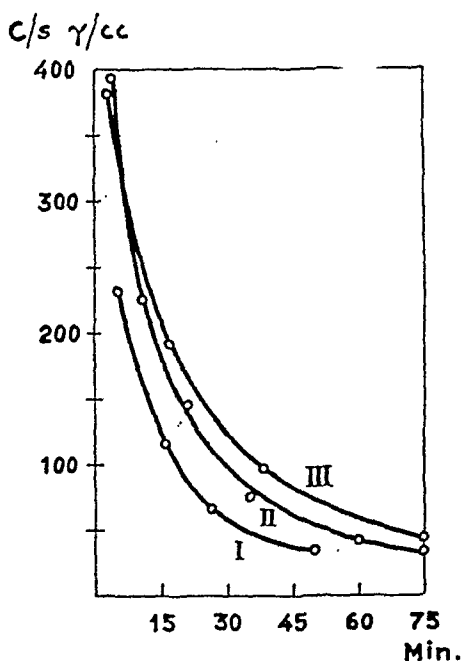


Fig. 12. The C/s during kidney perfusion.

- I. Cat, 2.6 kg. Pernocton 0.7 cc/kg. Perfusion fluid 260 cc heparinated blood (with about 35 % Tyrode's sol.). Addition of 86 mg Ci. Lively urine secretion during the whole experiment, about 17 mg Ci, or 20 % of the amount supplied, being excreted.
- II. Cat, 4.4 kg. Pernocton 0.7 cc/kg. Perfusion fluid 260 cc heparinated blood (with about 25 % Tyrode's sol.). Addition of 107 mg Ci. Urine secretion only at the outset, 5.3 mg Ci being then excreted.
- III. Cat, 3.6 kg. Urethane 1.6 gm/kg. Perfusion fluid 300 cc heparinated blood (with about 35 % Tyrode's sol.). Addition of 107 mg Ci. Only insignificant urine secretion to begin with.

circulation through the kidneys could be left, practically speaking, uninterrupted. All lateral branches and the vessels to the suprarenals and left spermatic vein were carefully tied. The urine was collected through a cannula tied into the top of the bladder.

Whether or not secretion of urine will be able to proceed appears to

depend to a high degree on whether volume of flow per minute and pressure are correctly adjusted at the outset of the experiment. This secretion obviously plays a minor part in experiments on the Ci-oxidation in the renal parenchyma, but when the effect of different factors on the Ci excretion has to be studied the flow rate and pressure must be stabilized at the start and then kept constant.

Fig. 12 gives quantitative data from three experiments with a single bulk addition of Ci. Even if urine secretion has ceased, so that no Ci can disappear by that path, yet Ci is rapidly eliminated from the blood. This shows that the Ci-oxidation in the renal parenchyma is very active. The conversion continues at a relatively high rate even down in the low concentrations, and one is therefore entitled to assume that it is the kidneys which keep the C/s at a constantly low level, and that the oxidation of Ci there is alone sufficient to account for the body's power to deal with the Ci supplied to it.

The amount of Ci oxidized in the renal parenchyma is most accurately calculated on the basis of experiments with *continuous* administration of Ci, as the C/s is not then raised excessively. A comparison of three experiments, which were carried out under the same conditions to ensure fully comparable results, gave a value of 32 γ per minute per gram kidney when the C/s averaged 90 γ per cc.

The cat kidneys were perfused with heparinated blood with 20–25 % addition of Tyrode's solution. After a preliminary period, when the blood flow per minute and pressure had been stabilized, a sample of the perfusion fluid was taken, whereupon sodium-citrate solution equivalent to 40 mgm. of Ci was added in uniform drip during 20 minutes. A fresh sample was then taken; the C/s at beginning and end of the Ci-drip is stated under the heading "Ci-conc." in the following report of the experiments.

	Min. Vol. cc	Pressure mm Hg	Ci-conc. γ /cc	Wt. of Kidney	Ci Consump- tion in mgm	Ci Consump- tion γ p. min. p. gm
I	58	80	25.7–160	31	18.4	29.7
II	54	90	36.5–123	27	17.2	31.8
III	60	65	63.5–136	43	29.7	34.5

In order to verify that the kidneys *in situ* play the same part in the normal Ci-metabolism, I drew a series of samples from the arterial blood and the renal vein at the same time. In most cases the blood was taken direct by puncture of the renal vein,

in a couple of cases from the left ovarian vein, which during pregnancy is well developed. The result is given in the following table, the differences being calculated in percentages of the arterial blood values.

Animal	Arterial	Renal V.	Diff. %	Animal	Arterial	Renal V.	Diff. %
Rabbit	111	78.2	29.6	Cat	51.2	42.3	17.4
»	89.7	65.9	26.5	»	64.9	56.0	13.7
»	127	78.4	38.3	»	69.0	54.0	21.7
»	103	71.0	31.1	»	55.0	42.1	23.5
»	83.9	66.3	26.5	»	56.4	44.6	20.9
»	108	73.1	30.5	Dog	53.2	37.0	30.5
»	80.8	55.0	31.9	»	80.7	46.4	42.5
»	68.9	44.8	35.0	»	57.6	25.5	55.7 ¹
»	68.3	43.7	36.0	Rabbit	46.5	36.0	24.3 ¹

¹ By the pentabromacetone method.

The tests show that there is a marked difference between the Ci-concentration in arterial and renal-vein blood even under normal conditions, when the C/s has not been raised by administration of Ci and the Ci-excretion is consequently very small. The difference is greater in the rabbit, about 30 %, than in the cat, about 20 %. But this is counterbalanced by the relatively much smaller weight of the kidney in the rabbit, on which account a lower renal blood flow per unit time has also to be taken into consideration. As a matter of fact, the Ci-consumption per minute per gram kidney of the rabbit may be regarded as being of the same order as that of the cat. This is shown by the experiments described below, in which the Ci-oxidation was evaluated on the basis of the Ci-differences found in arterial and renal-vein blood and the volume of blood passing through the kidneys per minute.

Attempts to perfuse isolated rabbit kidneys with the same technique as for the cat failed on account of vasoconstriction. The Ci-consumption was therefore measured in rabbit kidneys in the following manner: Into the right jugular vein of a heparinated rabbit a coarse, bent cannula was inserted and connected by a rubber tube with a cannula that fitted into the left renal vein. The connexion was filled with Ringer's solution. The renal vein was ligatured, the cannula placed quickly in, whereupon circulation could immediately commence at the anastomosis. A T-tube

was fitted in the latter for sampling. Immediately after, a sample was taken from the carotid artery. The flow volume *per minute* was measured by allowing the blood to flow out into a measuring-glass and noting the number of seconds required for 5 cc. to collect; the blood was afterwards re-injected. This technique is successful with rabbits, but in cats a transudation is likely to occur under the renal capsule. As the experiments were not originally intended for calculation of the absolute Ci-consumption, no haematocrit reading was made. In the following approximate calculation the Ci-content of the blood was considered to be 70 % of the C/s; the Ci-excretion in the urine is so small that it can be neglected.

	A. carot. C/s γ /cc.	V. renal C/s γ /cc.	Min. Vol. cc.	Wt. of kidney gm	Ci-consump- tion p. min. p. gm.
Rabbit I	91.0	69.4	17	12	21.4
» II	107	82.6	43	17	43.2
» III	96.4	76.2	23	9	36.1
» IV	151	116	11	7.8	34.5

The Citric Acid Content of Serum after Nephrectomy.

Previous experiments would lead one to expect a rise in the C/s if Ci-elimination via the kidneys were prevented. Such proved to be the case, as can be seen from the curves in *Fig. 13*.

The animals were under narcosis all the time. Nephrectomy was performed by way of laparotomy or from the back. The blood samples from rats were taken from different animals at varying lengths of time after ligation of the renal vessels, since continuous samples of sufficient size cannot be drawn from the same animal.

The curves show that in rabbits and rats the C/s rises very rapidly up to four or five times the normal value, while in cats the rise is much less, only double the normal figure. The cause of this may be that after the C/s has reached a certain elevation the liver of the cat, having greater Ci-oxidizing power, is able to maintain an equilibrium between Ci-formation and Ci-breakdown. There is also the possibility that an acidosis sets in and counteracts the rise.

The narcotized rabbits died only three or four hours after the nephrectomy, in spite of the fact that only a few blood samples had been drawn. The narcosis must in some way be responsible for this, for, if the kidneys are extirpated under ether narcosis and the rabbits afterwards allowed to revive,

they survive five to seven days. Then, however, first comes the postoperative or postnarcotic fall of the C/s observed both in man (SCHERSTÉN, 1931) and rabbits (GRÖNVALL, 1937), and after that the rise takes place much slower than in narcotized animals: the same rise will take days instead of hours (Fig. 14).

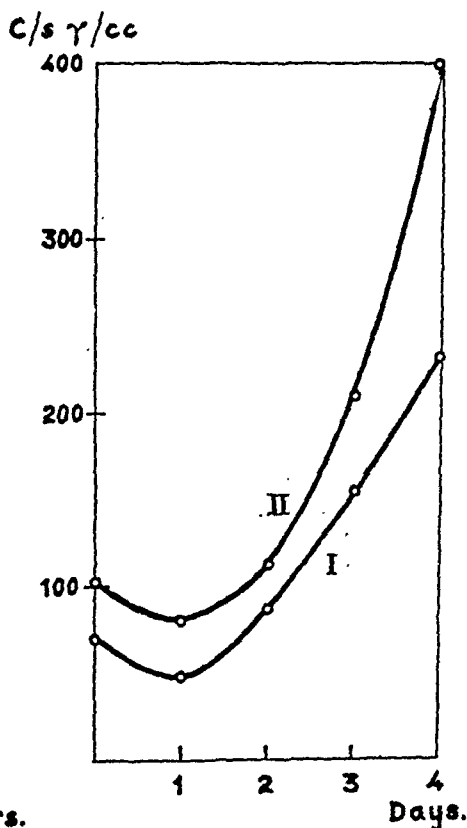
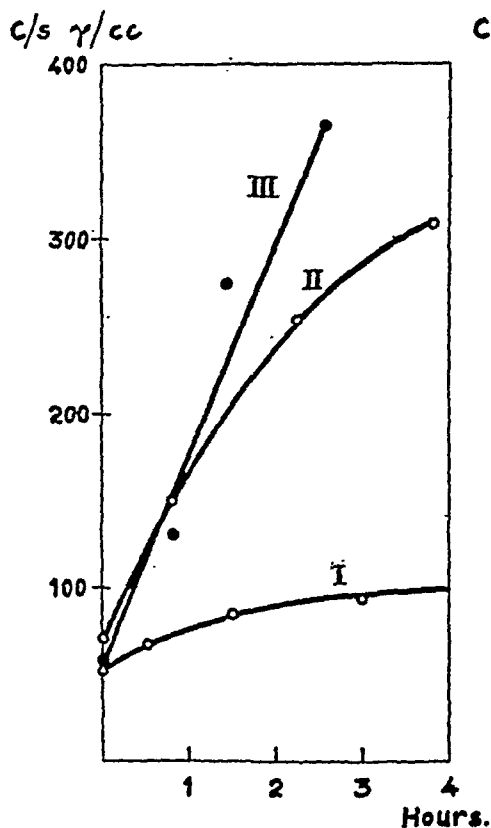


Fig. 13. The C/s after nephrectomy, narcotized animals.

- I. Cat, 3.6 kg. Urethane 1.25 gm/kg.
- II. Rabbit, 2.7 kg. Pernocton 0.95 cc/kg.
- III. Rats, 0.2 kg. Ether narcosis.

Fig. 14. The C/s after nephrectomy, non-narcotized animals.

- I. Rabbit, 4.2 kg.
- II. Rabbit, 6.2 kg.

The disturbance in metabolism brought about by nephrectomy is evidently very strongly accentuated by the derangement narcosis involves. Narcosis is stated to give rise to a certain amount of acidosis and an elevation of blood urea; furthermore, after nephrectomy blood urea and other non-protein compounds have been observed to increase more rapidly in dogs under narcosis (quoted from BEECHER, 1938). ANDON and

GISSELSSON (1938) observed the same difference between narcotized and non-narcotized animals in respect of creatinaemia, phosphataemia, and acidosis after nephrectomy.

The Citric Acid Concentration in Various Tissues Normally and After Administration of Citric Acid.

My experiments have shown that the Ci-oxidation is effected mainly in the kidneys, while the liver has little and the muscle no power to break down Ci. This is in conflict with the results of numerous works on Ci-metabolism in minced tissues (*inter alia* LANGECKER 1934, BREUSCH, 1937), according to which the Ci-metabolism is greatest in liver, less in kidney and muscle. Later, KREBS and EGGLESTON (1938) have shown that the breakdown of Ci in muscle, where the iso-citricodehydrogenase is very labile, is under optimum conditions still greater than in the liver, which further accentuates the lack of agreement between *in-vivo* and *in-vitro* experiments. However, it must be borne in mind that the dehydrogenase reactions are *reversible* and that in the intact cell they may be differently linked than in the injured cell. Another possibility is that the cells of the intact organ have plenty of other fuel to attack before beginning on Ci.

In connexion with these discrepancies between experiments on intact and minced tissues, the most important question is, however, whether the substance in question is able to penetrate into the uninjured cells. This, I have endeavoured to discover, so far as Ci is concerned, by investigating the Ci-content of the tissues before and after administration of Ci to the animals.

For the Ci-estimations I used the pentabromacetone method according to PUCHER, SHERMAN and VICKERY (1936), and followed their directions for the treatment of the tissues. These authors give the following Ci concentrations in tissues from dogs: kidney 12 γ per gm., muscle 11 γ , and liver only 1 γ . However, I soon found that the Ci-value obtained when the tissues were immediately immersed in trichloroacetic acid and pounded very rapidly was materially different from that obtained when they were first cut up with scissors or treated less rapidly. This applies especially to liver and kidney; for muscle it plays a minor part. In a piece of liver that had been finely cut during one minute, the Ci-concentration fell from 19 γ per gm. to 8.5. This may explain why LANGECKER (1934) could not find Ci in the tissues and why, as a general rule, such low concentrations have been found in the liver — the finding that is accepted generally as evidence that the chief seat of the body's Ci-oxidation is the liver. ORTEN

& SMITH (1937) froze down the tissues for an investigation on rats, which procedure ought to be effective, but as the animals were first bled to death, these authors may nevertheless have obtained too low values. If the animals are killed and the circulation through the organs is thus cut off before samples are taken, I found that the concentration in liver and kidney falls considerably in even a couple of minutes. (In one such experiment the amount of Ci was reduced from 26.5 γ to 12.7 γ per gm. in the liver, from 77 γ to 25.7 γ per gm. in the kidney.) I therefore took the specimens of the organs with circulation undisturbed or else took them practically simultaneously with the ligation of the vessels. They were dried and squeezed as free as possible from blood in a compress before being immersed in the trichloroacetic acid and ground with sand. The kidneys were first clipped in two, so that the capsule could be quickly removed and the pelvis dried free from urine. When two series of samples were taken from the same animal, a whole liver-lobe, a kidney and the thigh-muscle were taken from one side in the first round, and tissues from the other side, which were thus still quite intact, in the second round.

Some illustrative results from these estimations of the Ci-concentration in liver, kidney and muscle before and after administration of Ci have been collocated in the following table. The value for blood serum is also given as a measure of the Ci-concentration in the medium with which the cells are washed and have to enter into equilibrium. (The Ci concentration is given in γ per gram).

	Blood Serum	Liver	Kidney	Muscle	Ci-addition
Rabbit I	46.5	19.0	88.0	19.5	None
» II	63.5	26.5	77.0	22.3	»
» III	325	48.0	570	57.7	100 mg/kg
» IV	940	131	945	64.0	132 »
	720	65.0	680	50.8	(20 min. later)
Cat I	54.8 ¹⁾	25.3	55.8	29.2	None
	640 ¹⁾	93.8	2300	73.1	120 mg/kg (27 min.)
» II	800	136	1860	60.4	100 mg/kg
	464	54.1	1300	58.8	(20 min. later)

¹ Estimated by the THUNBERG method.

It will be seen that the Ci concentration in liver and muscle is normally lower than in blood serum, while in kidney it is the same or higher. Following addition of Ci the Ci-content of liver and muscle rises only slightly in relation to the C/s.

(It may be regarded as doubtful whether the increase found is much higher than is accounted for by the Ci-content of the blood left in the tissues). This also applies if the C/s has been elevated for a relatively long time, such as in three of the above-recorded experiments. On the other hand, the concentration in the organs declines with falling C/s, although this is still very high. The position is otherwise with the kidneys. In rabbits the Ci-concentration increases at least as much as in serum, and in cats a very powerful accumulation of Ci takes place in the renal tissue.

As to how the re-absorption conditions for a substance oxidized in the renal parenchyma itself are to be conceived in detail, I do not venture here to take up a position. It is however tempting to suppose that the ability of the renal tissue to oxidize Ci becomes a limiting factor for the re-absorption. This ability is obviously augmented with rising Ci-concentration, but when it is exceeded, Ci accumulates in the renal parenchyma in such quantities that re-absorption is hindered and large amounts pass out with the urine.

Since the Ci-concentration in both urine and renal parenchyma is considerably lower in the rabbit than in the cat, it is conceivable that Ci filters out at a lower rate in the rabbit, even if the C/s is the same.

Manifestly the high Ci-concentration in the renal tissue may to some extent depend on Ci-rich urine being left in the uriniferous tubules, in spite of the pelvis having been cut away and the tissue squeezed out, but this residue can scarcely be so large that it can even approximately explain the Ci-increase.

Heart Muscle. This differs very materially from ordinary muscle, in that *the Ci-concentration is normally considerably higher than in the serum.* After Ci-administration, however, the Ci of heart muscle does not increase in any high degree.

	Normal C/s γ /cc	Heart Muscle γ /gm		C/s after Ci-addition	Heart Muscle γ /gm
Rabbit	42.7 ¹)	125	Rabbit	325	250
»	66.0	265	»	720	320
»	61.5	233	Cat	640	206
Cat	43.5	130	»	464	203

¹ Estimated by the THUNBERG method.

In one case the Ci of a working rabbit-muscle was determined. A group of dorsal thigh muscles, which had to work against an elastic resistance, were excited from the nerve to make 45 jerks per minute during 30 minutes. The Ci-concentration was 39 γ per gm., in the corresponding group at rest on the other thigh it was 41.8 γ per gm., thus no sure difference.

From the experiments the conclusion may be drawn that *Ci has little or no power of penetrating into intact hepatic or muscular tissue*. Certainly the low concentration in the organs after addition of Ci might be due to a rapid oxidation of any Ci that had intruded (as is proved to be the case in the liver), but, since liver or muscle tissue removes no Ci or very little from the blood in perfusion experiments, in which Ci is offered to the cells in so physiological a way as via the capillaries, the conclusion put forward above may be regarded as well-founded.

Ci penetrates very readily into the renal parenchyma, but it must be remembered that its path there is via a re-absorption through the tubular epithelium, since the concentration in the parenchyma may be so much higher than in the blood. Still, judging from perfusion tests, it should be remarked that the oxidation of Ci in the renal tissue appears to be equally active even when secretion of urine ceases.

The rapid fall in the C/s after intravenous injection of Ci (Fig. 7) seems to receive an adequate explanation from these experiments: In addition to the large Ci-excretion in the urine when the C/s is high, there is this large accumulation of Ci in the renal parenchyma with its resultant higher rate of oxidation.

These experiments to determine the ability of Ci to penetrate into the tissues would also appear to have provided a satisfactory explanation of the discrepancy between *in-vivo* and *in-vitro* experiments. For I have not found any indications that the Ci formed in the body behaves differently from that supplied from without. That in respect of diffusion the endogenous Ci does not differ in behaviour from the exogenous is evidenced by the following experiment, in which an elevation of the C/s was brought about by means of bilateral nephrectomy.

22. 3. 40. Rabbit, 4 kg. starved 48 hours. Urethane 1.25 gm. per kg. Bilateral nephrectomy. After 2½ hours samples were taken from liver, muscle, and arterial blood and were analysed by the pentabromacetone method. The C/s had risen to 260 γ per cc., but the concentration in the liver was only 78.5 γ per cc. and in the muscle 52 γ .

LYNEN and NECIULLAH (1939) have studied the breakdown of succinic acid, malic acid and Ci by yeasts. They found that a number of earlier disagreements may be reconciled by taking into consideration the fact that the wall of the yeast-cell is but little permeable to these acids. On the other hand, according to HARRISON (1939), succinic acid penetrates readily into a frog's sartorius, although it does not undergo consumption there. And LUNDSGAARD (1938) found that alcohol is not consumed on muscle perfusion, although it makes its way into the cells and these contain the specific dehydrogenase.

Discussion of the Experimental Results ¹.

The Ci in the circulating blood has a definite tendency to remain at a constant level. There accordingly exists a constant equilibrium between the Ci-intake and Ci-output of the blood.

¹ After this work had been written, a preliminary report was published by DICKENS (1940), who seems to have opened up new fields of inquiry respecting the significance of Ci in the body under normal and pathological conditions. While investigating the Ci-concentration in different tissues DICKENS found that most of the body's Ci is contained in the skeleton, in which it may amount to not less than 1000 mgm. per 100 gm. of the dry, de-fatted substance. Thus there is here a large store of Ci, presumably as Ca salt. This discovery adds interest to the works dealing with the relation between Ci-metabolism and calcium metabolism, especially the conducive influence Ci has on the retention of calcium (e. g. WESTERLUND 1931, LANFORD, 1939) and with the beneficial effect Ci has on experimental rhachitis (e. g. HATHAWAY and MEYER, 1939). It also provides us with some new points of view respecting the use of Ci as an addition to cow milk in artificial feeding and, in general, as a beneficial agent in early infancy (see SRWE, 1938). It remains to be seen whether the Ci in the skeleton takes part in the constant Ci-balance in the circulating blood. The conditions under which my experiments on eviscerated preparations and my hindlegs perfusions were carried out do not exclude the possibility that the Ci which I thought came from muscle actually came from the skeleton instead. As to the rest of my experiments, a possible flow of Ci from the skeleton cannot play any part. DICKENS further found that malignant tumours from mice, rats and rabbits contain much more Ci than normal tissue and that embryonic tissue does so too. Considered along with SCHERSTÉN's finding that the male gonads are very rich in Ci, this may suggest that a high Ci concentration occurs where a brisk new-growth of tissue elements is going on. The fact that the C/s is higher in foetuses and young animals has already been demonstrated by GRÖNVALL (1937).

In tests with the enzymic method THUNBERG (personal communication) has not only confirmed DICKENS' find respecting Ci in bone, but found still higher values (up to 2 %).

According to the experiments reported here, the *Ci* is mainly supplied from the portal area and from the muscles, while it is eliminated to a large extent by the kidneys and to a small extent by the liver, more in cats than in rabbits. Tolerance tests show that the body's power to transform *Ci* supplied from without is much greater than is normally called on. My experiments have been largely carried out on isolated organs, but I have consistently tried to verify that these organs when *in situ* play the same part in the *Ci*-metabolism. When *Ci*-oxidation has been referred to here, this has not meant, of course, the complete combustion of *Ci* to carbon dioxide and water, but only its breakdown into some other compound. I have not registered the influence of *Ci* on the O_2 -consumption or the respiratory quotient of the organs.

The object of the following account is to put my experimental results into relation with that conception of the formation, breakdown and significance of *Ci* in the body which has appeared in the literature of recent years.

KNOOP and MARTIUS (1936) consider that *Ci* can be formed from oxaloacetic acid and pyruvic acid, and they hold it probable that the synthesis takes place *in vivo* in the same way. This has been corroborated by BREUSCH (1939). The *Ci*-breakdown is assumed to occur in the following manner (MARTIUS, 1937, 1938): *Ci* is first transferred to the unsaturated *cis-aconitic acid*, which takes up water again and forms *isocitric acid*. This is dehydrogenated to *oxalosuccinic acid*, which spontaneously gives off CO_2 and forms *α -ketoglutaric acid*, which under decarboxylation and dehydrogenation is converted into *succinic acid* and later via *fumaric acid-malic acid* into *oxaloacetic acid*.

I have not gone here into the question of from what *Ci* is formed and what the more immediate products of its breakdown are in the body. To give reliable results an investigation into this aspect would demand parallel determinations of several of the above-mentioned metabolites, for which there are only tedious analytical methods that are not sufficiently accurate for the concentrations normally occurring in the body. Moreover, there would first be required a similar investigation into the normal metabolism of these acids in the body as that here accorded *Ci*, this as a safeguard against false interpretations.

The chain of *Ci*-breakdown products would thus include all the acids which were shown by THUNBERG 30 years ago to have a distinctive position in the metabolic processes of minced muscle. They have since generally been regarded as disintegration stages which, in the course of oxidation,

are continually being formed and continually being broken down again. Through the work of SZENT-GYÖRGYI and collaborators (1935), however, C_4 -dicarboxylic acids are assigned another metabolic rôle: These acids are thought to act as catalysts in the oxidation of carbohydrates, forming an intermediary link for the transport of hydrogen to the cytochrome system. In this they are conceived as reversibly swinging between the hydrogenated and dehydrogenated form, without entering themselves into the chain of reactions. SZENT-GYÖRGYI's theories, however, involve certain difficulties of interpretation, as was pointed out e. g. by MARTIUS (1939). Later, KREBS and JOHNSON (1937) set up another theory, based on the new conception of the formation and breakdown of Ci. According to this — the "citric acid cycle" theory of KREBS —, Ci is formed in the muscles from oxaloacetic acid and a product of carbohydrate metabolism, a triose or similar combination. When Ci is broken down later, oxaloacetic acid, according to the course of reaction outlined above, is rebuilt and with triose forms Ci again. In this way a cyclic process is maintained, the result of which is that the carbohydrate participating in the reaction undergoes complete combustion. Accordingly, it is here thought that the reaction proceeds in only one direction and that Ci as well as the C_4 -dicarboxylic acids take part in the chain; thus the theory differs in principle from SZENT-GYÖRGYI's. On these lines one would get a plausible explanation of the extremely high activity of the citricodehydrogenase and of its universal occurrence in the cells. The low but rather constant Ci-concentration in blood and tissues — in man scarcely more than 0.0001 mol. — would also seem to argue in favour of a catalytic function. The Ci-cycle theory is also further supported in recent works of KREBS (1937), KREBS, SALVIN and JOHNSON (1938), and KREBS and EGGLESTON (1938). The last-mentioned authors think that insulin has its point of attack in this Ci-cycle. The theory has however met with opposition from several investigators, e. g. BREUSCH (1937, 1939), BANGA, OCHOA and PETERS (1939), THOMAS (1939). STADIE, ZAPP and LUKENS (1940) were not able to confirm the catalytic function of Ci or the insulin effect on the Ci-cycle. No conclusive evidence for either of these theories seems to have been advanced as yet.

On the basis of my experiments no attitude can be directly taken up towards these theories based on *in-vitro* experiments. Any Ci metabolism that might be going on *within* the cells would not necessarily find expression in my experiments. Even if Ci cannot pass through the cell-wall, or can only do so with difficulty, a Ci-formation is conceivable from substances that can pass through. On the other hand, the cell-wall may be permeable to cleavage products of Ci but the Ci-metabolism itself may go on within the cells and need not become apparent in perfusion tests. In this way the cells can nevertheless have use for their highly active enzyme.

This question, as a matter of fact, is very difficult to solve: by perfusion experiments one cannot expect with any certainty to get at intracellular processes, by *in-vitro* experiments on minced tissues one can obtain a good idea of the metabolic *possibilities* possessed by the cells but no reliable knowledge of how the cells make use of these possibilities, as they are seated linked together in an intact complex. Moreover, a considerable part of the enzymic potentiality of the cells is doubtless only called upon in pathologic situations, e. g. autolysis.

However, I have been able to take up a position with regard to a series of *in-vivo* experiments which have been taken as evidence confirming the citric-acid-cycle theory of Krebs. ORTEN and SMITH (1937 a, b) were able to show that the Ci-excretion in the urine of dogs and rats increases immensely after intravenous injection in large amount of certain metabolites, chiefly malic acid, fumaric acid, and succinic acid. But such substances as malonic acid and maleic acid also raise the Ci-excretion in the same degree. Later experiments (ORTEN and SMITH, 1938 a, b, 1939) led these authors more and more to the opinion that the Ci is formed from the injected substances and that this formation takes place chiefly in the kidney or is dependent upon the presence of the kidney. The kidney was thus assigned a rôle in the Ci-metabolism that is the direct opposite of the one indicated by my experiments. ORTEN and SMITH's experiments were fully confirmed by KREBS, SALVIN and JOHNSON (1938) and were accepted by these authors as proof that it is possible to demonstrate the occurrence of the citric acid cycle in the living organism. However, by determining the Ci-concentration in samples taken at the same time from the arterial and renal-vein blood, and by means of kidney perfusion tests, I was able to show *that Ci is not formed in the kidneys after administration of malic acid, but that this acid inhibits the normal breakdown of Ci in the renal parenchyma and thereby brings about a powerful increase in the Ci-excretion in the urine* (MÅRTENSSON, 1939). It is this increase in the excretion of Ci, caused by the inhibition of the Ci-breakdown in the kidneys, that has been interpreted as a formation of Ci from the injected substances. Consequently, *the in-vivo experiments cited above cannot be accepted as proof of the accuracy of the Ci-cycle theory of Krebs.*

The effect of the injected substances on the Ci-excretion is easier to understand in the light of my later experiments, which

show that the Ci-concentration in the renal tissue is high and increases markedly on administration of Ci. It is very possible that the other metabolites accumulate in the renal tissue in the same way as Ci. In the works cited they were supplied in very large doses, and it is therefore easy to understand that they ought to have a strong effect, whether this effect is interpreted as operating through their specific adsorption to, and blockage of, the isocitricodehydrogenase (e. g. the effect of malonic acid on succinodehydrogenase, QUASTEL and WOOLDRIDGE, 1928) or through the dehydrogenating capacity of the renal parenchyma being quite insufficient to cope with the Ci when this tissue is charged with some other readily combustible substance to a high concentration. Favouring the first alternative is the fact that a pure "enzyme poison" such as malonic acid has this effect; it also inhibits the oxidation of Ci in the tissues (BREUSCH, 1937). Another support for this alternative is the somewhat similar constitution of malic acid and isocitric acid, as was pointed out to me by LEHMANN, who (1938) investigated similar problems relating to the lactico-dehydrogenase.

In experiments on the dehydrogenation of Ci by cucumber-seed extract I have found a retardation of the MB-decolorization to follow addition of l-malic acid in concentrations not exceeding those in which malonic acid causes an inhibition. This is mentioned as a parallel. In order to prove anything the experiments would have to be carried out with isocitricodehydrogenase from kidney.

Since my first Ci-work appeared (MÅRTENSSON, 1938), in which the Ci-concentration was shown to be lower in the renal-vein blood than in the arterial blood, SMITH, ORTEN, JOHNSTON and BANGUENESS (1939) have conducted similar experiments on dogs. Conformably with their conception of the course of events, they had expected to find, under the influence of malic acid, a higher concentration of Ci in the renal-vein blood than in the arterial blood, due to a "reverse leakage" similar to what occurs in the case of ammonium. They found the converse, a lower concentration in the renal-vein blood, both before and after malate injection. This is accordingly a good confirmation of my results, the more valuable because the experiments were carried out on non-narcotized animals with the kidney brought subcutaneously forward. On the other hand, in a work of SMITH and MEYER (1939) on the influence of diet on the endogenous production of citric acid, all these meta-

bolites, whose influence on the Ci-excretion has been studied, are spoken of as "definitely recognized precursors of citric acid", and SIMOLA and KOSUNEN (1938) are stated to have extended this series of precursors to pyruvic acid, α -ketoglutaric acid, and glutaric acid. SIMOLA (1938) nevertheless emphasizes that the effect produced by these substances may depend upon their preventing further cleavage of Ci, which therefore accumulates.

My investigations would appear to have shown that *all these works on "the precursors of the endogenous citric acid" are in no way conclusive*, since their results may be entirely due to a displacement in proportion between the Ci broken down in the renal parenchyma and that excreted in the urine. In fact, it is for the present an unsolved question whether certain of these substances in experiments *in vivo* give rise to a Ci-formation elsewhere than in the kidneys. That possibility manifestly remains open, and for oxaloacetic acid and pyruvic acid is fairly probable judging from *in-vitro* experiments (MARTIUS, 1937; BREUSCH, 1939). Similarly, it must be said that *all works on the influence of diet on the endogenous Ci are not very convincing* so far as they postulate the derivation of Ci from a particular kind of food (e. g. FÜRTH, MINNIBECK, and EDEL, 1934; SHERMAN, MENDEL and SMITH, 1936; VERKADE, 1938, quot. from SMITH and MEYER, 1939). The fact is that the diet, without giving rise to Ci, may have an influence on the quantity of Ci in the urine (1) by affecting a bacterial Ci-production in the intestine, (2) by direct action on the Ci-oxidation in the renal parenchyma, and (3) by indirect action in the form of a displacement of the acid-base equilibrium. These complicated factors may, on one hand, entirely conceal, on the other, powerfully accentuate, any Ci-formation going on from the food.

BREUSCH (1939) found that some Ci was formed in minced kidney after addition of oxaloacetic acid. He interprets this as a mechanism by which the body gets rid of surplus C₄-dicarboxylic acids, which are not themselves excretable in the urine, and by which it keeps the concentration of these important acids constant in the tissues. Such an explanation would also cast more light on the import of ORTEN and SMITH's results. This broad theory of *in-vivo* conditions founded on an *in-vitro* experiment loses however much in force in view of: (1) the fact that in the same work it is shown that fumaric acid-malic acid cannot give rise to Ci, (2) the results submitted here from my experiments, (3) the fact that at least the succinic acid is excreted in the urine just as readily as citric acid (FÖRSSMAN, unpublished).

Among the breakdown products of Ci, α -ketoglutaric acid was stressed by MARTIUS (1937) as being of special importance because it can be a mother substance for important amino acids. VON EULER, ADLER, GÜNTHER and DAS (1938) could show that α -ketoglutaric acid is able under physiological conditions to bind ammonia and form glutamic acid in presence of the specific enzymes. This reaction is so much the more important as the amino group of the glutamic acid can be transferred by enzyme action to other α -keto acids (BRAUNSTEIN and KRITZMANN, 1937) and the α -ketoglutaric acid then reformed can afterwards take up fresh ammonia. We can thus speak of a catalysis of the linking of free ammonium, with formation of amino acids. ADLER, v. EULER, GÜNTHER and PLESS (1939) consider it probable that direct coupling of the Ci breakdown with the synthesis of glutamic acid is the most effective path for the formation of amino acids. Ci provides not only the substrate, α -ketoglutaric acid, but also the hydrogen for the synthesis of glutamic acid. Since both isocitric dehydrogenase and glutamic dehydrogenase are present in all animal tissues, these authors hold it probable that the process is a universal cellular reaction in the body. In favour of this function of Ci argues especially the fact that the breaking down of Ci occurs chiefly in the renal parenchyma, which occupies a central position in amino-acid metabolism. The continual supply of Ci to the kidneys would appear to be also quantitatively sufficient to have some effect; calculating on the basis of the conditions in man, about 10 grams would be daily transformed in the kidneys. This suffices perhaps for the formation of the greater part of the so-called dispensable amino acids, which may be absent in the food, as the body itself is able to build them up from other material. The question as to whether this connexion between amino acids and Ci can be experimentally demonstrated *in vivo* is dealt with in the next chapter.

According to KREBS and COHEN (1939) this formation of glutamic acid from α -ketoglutaric acid and ammonia is not of great importance in other tissues than renal cortex and heart muscle. These tissues according to my experiments have normally a high Ci-concentration, which may also speak in favour of this connexion between Ci and amino acids.

Summary.

Following a review of previous investigations on the citric-acid metabolism in animal bodies, an account is given of a series of experiments which have yielded the following results:

The citric-acid content of blood serum (C/s) is considerably higher than that of the red blood corpuscles (probably five or six times higher).

Intravenously injected citrate (Ci) is rapidly eliminated from the blood (up to 100 mgm per kg disappear almost completely in 1 $\frac{1}{2}$ hours). The fall in concentration is the more rapid the higher the serum Ci-level is.

The liver takes only a very small part in this Ci-elimination, which is shown by perfusion of the isolated liver, by Ci-injection direct into the portal vein, and by analysis of joint samples from the portal and hepatic veins.

It is confirmed that orally administered Ci is rapidly absorbed. Even under fasting conditions, however, the C/s is higher in the portal than in the arterial blood, which is considered to be due to formation of Ci during metabolic processes in the intestine or through bacterial action; experiments show the latter to be operative in rabbits.

After a functional cutting-off of the liver and portal area, the C/s falls in the rabbit (owing to the prevented Ci-absorption from the intestine outweighing the power of the liver to break down Ci) but remains fairly unchanged in the cat, and the body is still able to remove intravenously injected Ci from the blood.

Ci is formed in the muscles, for the C/s is found to rise in an eviscerated preparation as well as in a perfused, isolated hind-leg preparation, and the venous blood coming from muscle has a higher Ci-concentration than the arterial.

After intravenous administration of Ci the urinary excretion of Ci is very greatly elevated so long as the C/s is high, but diminishes again as rapidly as the C/s falls.

Renal tissue has a great capacity to oxidize Ci, which is evidenced by perfusion experiments on isolated kidney. The Ci-concentration in the renal-vein blood is 20—30 % lower than in the arterial blood, even when the amount of Ci in the urine is very small. After nephrectomy there is a rise in the C/s.

The Ci-concentration in liver and skeletal muscle is lower than in the serum, but in renal tissue and heart muscle it is higher than in the serum. After administration of Ci the con-

centration rises inconsiderably in liver and muscle, whereas in renal tissue the rise is very substantial, which is regarded as proof that Ci is unable, or only able with difficulty, to penetrate into intact liver and muscular tissue. This explains the lack of agreement between *in-vivo* and *in-vitro* experiments respecting the oxidation of Ci.

The results of the experiments are discussed with reference to the more recent conception of the formation, breakdown and function of Ci in the body. It is stressed that the hitherto published *in-vivo* experiments on precursors to the endogenous Ci are not conclusive, since their results may depend solely on a displaced proportion between the Ci-oxidation in the renal parenchyma and the Ci-excretion. (Nor can they, therefore, be regarded as a corroboration of the citric-acid-cycle theory of KREBS). The same applies to experiments on the influence of food on the endogenous formation of Ci.

The fact demonstrated here that Ci is continually being supplied to the kidneys and oxidized there suggests that Ci may have a function to fulfil within the amino-acid metabolism.

CHAPTER III.

What is the Cause of the Hypercitricaemia in Lesions of the Hepatic Parenchyma?

THUNBERG (1933 a), on investigating the C/s in various pathologic states, found a raised value in, among other disorders, hepatitis. Oral administration of Ci to a hepatic subject was followed by a powerful rise of the C/s with a slow return to the initial value (THUNBERG, 1933 b). The extensive clinical studies of SJÖSTRÖM (1937) showed very clearly that the C/s rises so regularly in lesions of the hepatic parenchyma that determination of this value affords a very valuable aid to the differential diagnosis of hepatitis and obstructive jaundice.

This has subsequently been confirmed in clinical practice, in which the Ci-test has come into extensive use, at any rate in the Scandinavian countries. The C/s determination seems to have gained still greater value for differential diagnosis since it was combined with determination of the serum-phosphatase activity by a simple and reliable method (BUCH and BUCH, 1939), which renders possible a positive demonstration of an obstruction in the bile passages. But this latter test is also positive in a number of hepatic cases (about 10 % according to GUTMAN, OLSON, GUTMAN and FLOOD, 1940), and hence it is just the combination of the two tests that is so valuable.

From his animal experiments on these problems SJÖSTRÖM came to the conclusion that the amount of Ci-dehydrogenase in the liver is reduced in lesion of the hepatic cells, with the result that the normal capacity of the liver to break down Ci is diminished, this being considered the cause of the C/s rise. In clinical literature (BUCH, 1940) this explanation has been formulated thus: The Ci-dehydrogenase content declines considerably in parenchymatous diseases of the liver and therefore the Ci is not broken down in the usual manner but passes into the blood to a greater or less extent.

This very natural explanation cannot, however, be correct. Those perfusion tests in which SJÖSTRÖM found an immense decomposition of Ci in the liver have proved inexact and their results do not fit in with other known facts respecting the Ci-metabolism. SJÖSTRÖM was able to show that the C/s rises in hepatic injury produced by allyl formiate, but, obviously, this says nothing about the underlying mechanism. The perfusion tests on experimentally injured liver, which SJÖSTRÖM has also taken as a support for his conclusion, cannot be awarded any evidential value (even if the technique employed had been correct), for, in two reported experiments with heparinated blood, the Ci-metabolism was in one case only 1.5 %, in the other 2.7 %, lower than in an uninjured liver. As a matter of fact, rather would a higher Ci-metabolism have been expected in the injured liver, since, as SJÖSTRÖM asserts elsewhere in his work, the necrotic liver contains large amounts of enzyme, which should have a greater chance of coming into contact with its substrate than in normal liver.

Experiments of mine submitted earlier in this work have shown that in the rabbit and cat the kidneys play a considerably greater part than the liver in the Ci-breakdown, and it is evidently the kidneys which keep the C/s at a fairly constant value. Experiments on dogs and rats indicate the same condition there. It may accordingly be regarded as very probable that the Ci-metabolism in man behaves in the same way. Judging from animal experiments the renal oxidation of Ci rises with rising C/s, at least to begin with, so that the percentage difference between the Ci-concentrations in the arterial and the renal-vein blood remains about the same. If it is assumed that this also applies to man, then an assumption that the hypercitricaemia in hepatitis (which may have a value of over 100 γ Ci per cc) depends upon a cessation of Ci-oxidation in the liver would presuppose that this Ci-breakdown in the liver is several times greater than that in the kidneys. This is most improbable.

The situation, therefore, can scarcely be conceived otherwise than that the influence of the parenchymatous liver injury on the C/s is *indirect*. This is also suggested by the fact that the elevation of the C/s occurs early in the morbid process. To account for the rise two possibilities may be contemplated. Either the Ci-formation in the body is augmented under the influence of the profound change in metabolism that a grave

liver injury involves, or else this injury brings about an inhibition of the Ci-breakdown in the kidneys. Experiments with administration of Ci to hepatitics speak decidedly in favour of its being the Ci-oxidation that is reduced. A strongly retarded return of the C/s to its initial value ought not to occur if the hypercitricaemia depends upon an increased Ci-formation with an unimpaired Ci-decomposing capacity. If the renal oxidation of Ci is inhibited in some way or other, this is evidently compensated for by an elevation of the C/s to such a level that the Ci-breakdown becomes equal to normal when this new position of equilibrium has been established. That is to say, the absolute difference between the Ci-concentrations in the arterial and renal-vein blood is equal to normal, though if calculated as a percentage it is lower. In this connexion it may be mentioned that the absolute increase of Ci in the circulating blood when there is a rise of, say, 10 γ /cc in man, which is regarded as clearly pathologic, is not greater than about 0.05 gram, thus very low in relation to the power of the kidneys to break down Ci.

The breakdown of Ci in the renal parenchyma may no doubt be inhibited in several ways as a result of a severe injury to the liver-cells: (1) by accumulation of metabolic products which do not undergo further decomposition in a normal manner, (2) by the inability of the liver to deal with the substances absorbed from the intestine, or by such substances entering to a greater extent direct into the circulation, for instance, in cirrhotic conditions, or (3) by toxic products from the injured liver parenchyma itself. Clinicians speak of a "hepatonephric syndrome": the kidneys have to take over the detoxicating function of the body if the liver fails, but they do not possess the great margin of safety of the liver and fall short of the needs rather soon themselves. The toxic substances accumulate in the epithelium of the convoluted tubules, where they may cause considerable damage. In addition, a direct action on the renal parenchyma by the same toxic agent as injures the liver must be taken into consideration.

Without going into the clinical literature or other experimental works on the functional connexion between liver and kidneys, I am submitting here an account of a series of animal experiments that may throw some light on the mechanism underlying the rise of the C/s in injury of the hepatic cells. The experiments are grouped on the same lines as those reported in the preceding paragraph.

The C_i metabolism after functionally cutting off the liver and preventing absorption from the portal region.

In Chapter II (p. 48) an account is given of experiments devised to arrive at the C_i conditions after functional elimination of the liver and portal area. The C/s was found to fall considerably in the rabbit but to keep fairly constant in the cat. In the rabbit the C_i -breakdown after intravenous administration seemed to be fairly unchanged. A tolerance-curve (Fig. 9) shows, however, that the C/s attains an equilibrium at a rather high level, which is indicative of a certain amount of inhibition of the C_i breakdown in the kidneys, although this inhibition, in view of the rapid fall of the curve at the beginning, cannot be large.

In the following experiment on a rabbit, in which the liver and portal area were cut off from the circulation, the C/s difference between the arterial and renal-vein blood was only 14 % after 2 hours and 20 minutes, thus indicating a distinct inhibition of C_i breakdown. In all experiments of this type the C/s also shows a tendency to rise towards the end.

5. 4. 40. Rabbit, 2.7 kg. Urethane 1.25 gm/kg. Ligation of the arteries to the portal area as well as of the portal vein. Continuous injection of 10 % glucose, 1 cc in 10 minutes.

	Normal value	60	120	140 min. after ligation
C/s	71.9	27.3	28.4	28.7 γ/cc .

Corresponding experiments on the cat show no fall but, if anything, a small rise of the C/s (Fig. 9). This may be interpreted as indicating that in this animal the supply of C_i from the intestine and the C_i consumption in the liver keep in equilibrium with each other, but it may also be due to the fact that in the cat there sets in a considerably earlier and more effective inhibition of the renal C_i -breakdown than in the rabbit. Such an inhibition appears very clearly after addition of C_i to a cat prepared in this way.

26. 5. 38. Cat, 3.1 kg. Urethane 1.25 gm/kg. Ligation of arteries leading to the portal area as well as of the portal vein. During the experiment 1 gm of glucose was administered. Intraven. inj. of C_i , 42 mg/kg.

	Before C_i -inj.	2	18	38	55	125 min. after C_i -inj.
C/s	58.8	591	236	153	155	160 γ/cc .

7. 4. 40. Cat, 5 kg. Urethane 1.25 gm/kg. Same operation. Continuous inj. of 20 % glucose, 1 cc in 10 min. During one hour after ligation of portal area, the C/s rose from 31 γ to 42.2 γ/cc . After that inj. of C_i , 60 mg/kg.

	Before Ci-inj.	3	15	35	60	100 min. after Ci-inj.
C/s	42.2	854	510	399	321	282 γ /cc.

A comparison of the above elimination curve with one obtained from an intact cat (in which the kidneys also play the essential rôle in the Ci-oxidation), or with one giving the Ci breakdown in isolated kidneys, will clearly show that the *breakdown of Ci in the renal tissue must here have been subject to a pronounced inhibition*, since so moderate a dose of Ci, even after 100 minutes, still held the C/s up at a level seven times higher than the initial value.

The C/s in a sample taken from the renal vein at the same time as the last sample was, however, only 13.5 % lower than in the arterial blood, which certainly implies an inhibition, though scarcely so pronounced as might be expected in this case. Certain difficulties, however, arise in drawing conclusions from the difference between the C/s of the arterial and that of the renal-vein blood, unless the blood flow through the kidneys per unit of time is simultaneously measured.

The Ci-metabolism after functionally cutting off the liver but retaining the portal circulation intact.

The object of this type of experiment is to study the influence on the C/s of that part of a deficient liver-function which consists in the liver not being able to deal with the substances coming from the intestine, or in these substances passing direct into the circulation, as in conditions of cirrhosis.

The preparation technique was as follows: In the right jugular vein of the heparinated animal was tied a coarse, bent cannula, which was connected by a rubber tube with a cannula fitted into the portal vein. The system was filled with Tyrode's solution. The portal vein was ligated as closely as possible to the liver, then clamped peripherally, whereupon the cannula was quickly tied in and the circulation could immediately start in the anastomosis. Then the hepatic artery was ligated. In spite of continuous injection of glucose (150—200 mgm per kg per hour) the animal did not survive more than 1 $\frac{1}{2}$ —2 hours.

In these experiments with intact portal circulation, however, *there is a vigorous rise in the C/s* also in the rabbit. This must mean that substances absorbed from the intestine cause a further inhibition in the breakdown of Ci if they enter the circulation without first being dealt with by the liver. For the preceding type of experiment clearly shows that an increased

Ci-formation is not established if the liver is put out of function. The C/s rise seems to be less in animals that have been fasting rather a long time, i. e. in which the absorption of substances from the intestine cannot be very large.

The following experiments may be adduced:

2. 5. 38. Rabbit, 2.7 kg. Starved less than 24 hours.

Normal value	20	40	67 min. after op.
C/s 109	140	158	170 γ /cc

5. 4. 40. Rabbit, 3 kg. Starved 24 hours.

Normal value	60 min after op.
C/s 61.7	107 γ /cc

The rabbit died as a result of pulm. embolus after another 40 min. and before further samples had been withdrawn.

13. 11. 39. Rabbit, 3.9 kg. Starved 48 hours.

Normal value	30	60	90	140 min. after op.
C/s 61.9	78.5	84.5	97.3	112 γ /cc

20. 11. 39. Cat, 2.9 kg. Starved 24 hours.

Normal value	30	65 min. after op.
C/s 50.7	67.6	94.6 γ /cc

6. 5. 40. Cat, 2.7 kg. Starved 72 hours.

Normal value	30	60	90 min. after op.
C/s 46.4	47.3	51.2	64.3 γ /cc

In this case the rise of the C/s was not much greater than when the liver was cut off from the general circulation and the portal flow was arrested, but here there was obviously nothing that could be absorbed from the empty gastro-intestinal canal.

When Ci is administered to a cat provided with a portal vein-jugular vein anastomosis, the Ci-elimination is retarded and soon ceases at a high level, which shows that the breaking-down mechanism is effectively arrested.

7. 5. 40. Cat, 2.3 kg. Starved 24 hours. After the anastomotic circulation had been in action 20 min., Ci equivalent to 60 mgm per kg was injected for exactly five minutes.

Before inj.	3	15	35	60	90 min. after inj.
C/s 45.5	622	358	267	270	266 γ /cc

BOOTHBY and ADAMS (1934) investigated the urinary Ci-excretion of dogs following removal of the liver. After the first four hours this excretion rose considerably above the normal, which the authors interpret as indicating that possibly Ci in the dog is normally utilized or destroyed

by the liver. Instead of this, the rise probably depends on an arrest of the normal Ci-breakdown in the renal parenchyma following the hepatectomy, the amount of Ci in the urine being thereby increased. This increase stands out especially clearly in the dog, as the normal Ci-concentration in the urine of this animal is extremely low.

The Ci-metabolism in experimental lesion of the liver.

SJÖSTRÖM's work (1937) contains a survey of the technique for producing a liver injury by chemical means. In a couple of cases I duplicated SJÖSTRÖM's experiments, administering *allyl formiate* (Allylium formicicum, Schering-Kahlbaum) intraperitoneally to a rabbit. Macroscopically the liver thereupon presented a picture corresponding well with that described by SJÖSTRÖM from his experiments, and the C/s also rose moderately at the end. However, it is doubtless difficult in such experiments to keep the experimental conditions constant for any long period of time. In the rabbit the C/s varies rather much with the food, and, even if the latter is kept constant, the C/s drops considerably if the animal under the influence of the intoxication refuses to eat. Moreover, intraperitoneal injection of allyl formiate appears likely to cause an irritation leading to peritonitis, and such inflammatory states depress the C/s. It may be these factors that caused the incipient decline of the C/s which SJÖSTRÖM found in all his experiments, rather than an assumed increase of the Ci-dehydrogenase activity in the injured liver.

Any attempt to produce injury of the liver by chemical means will always involve the risk of directly affecting the renal parenchyma. In the experiments with allyl formiate, the kidneys appeared to be intact (at all events macroscopically). In an experiment with *chloroform* poisoning, in which 0.1 cc of chloroform per kg was daily injected subcutaneously, the rabbit was killed after eight days, and the kidneys then exhibited the same pronounced fatty infiltration as the liver. The serum samples were then markedly icteritious. The C/s behaved as follows:

	4. 4.	6. 4.	7. 4.	8. 4.	9. 4.	11. 4
C/s	107	96.3	122	105	124	130 7/cc.

A sample of the renal-vein blood, which was taken at the same time as the last sample from the carotid artery, had a value of 123 per cc. The small difference as against the arterial value points to a pronounced arrest of

the Ci-breakdown, although this may here be due to the direct chloroform action on the renal parenchyma. Chloroform does not, accordingly, give pure experimental conditions.

I therefore tried to produce a severe hepatic lesion without administering any toxic substance. With this end in view I utilized the condition demonstrated by McMICHAEL (1937) that the rabbit's liver obtains almost all its oxygen from the hepatic artery and that necrosis of the liver sets in if the hepatic artery is ligatured.

The following experiment shows how the C/s varies when the hepatic artery is ligated under ether narcosis and the rabbit is afterwards allowed to revive.

6. 12. 39. Rabbit, 5.1 kg. Lively just after operation, began to be sluggish 17—18 hours later, more and more so subsequently. Died after 28 hours.

	Normal value	7	17	24	28 hours after lig.
C/s	63.9	62.0	50.4	67.6	107 γ /cc.

First comes the usual post-operative decline in the C/s. This counteracts a possible rise due to the hepatic injury, which rise, however, makes its appearance at the end. Macroscopically the liver seemed to be necrotic throughout.

Seeing that the post-operative reaction disturbs the experimental conditions, I went over to allowing the animals to lie under light narcosis the whole time. Then there occurred the same thing as was observed in the experiments with nephrectomy: the course was run much quicker under narcosis, the animals dying in five or six hours after ligation of the hepatic artery. During this time *the C/s rose evenly up to double the value or higher.*

The following experiment is submitted as typical: 4. 4. 40. Rabbit, 2.6 kg. Urethane 1.25 gm/kg.

	Normal value	3	4	5 1/2 hours after lig.
C/s	60.7	85.2	99.8	115 γ /cc.

The blood pressure was somewhat inferior in the sample last taken. The rabbit died soon after withdrawal of this sample.

In Fig. 15 curve III gives the Ci-elimination found in an experiment in which Ci was administered after ligation of the hepatic artery. Ci was injected two hours after the ligature was applied, and after a further 2 1/2 hours the rabbit exhibited no strong effects from its liver injury. In spite of this, the

Ci-elimination is very much retarded. For comparison I am submitting a curve showing the elimination after addition of the same amount of Ci to a normal rabbit (Curve I) and to a rabbit with its liver entirely cut off from the general circulation (Curve II). The experiment shows that *for breaking down Ci in the body an injured liver is inferior to no liver at all*, a further support for the view that the influence exercised by hepatic lesion on the Ci-metabolism is indirect.

Broadly viewed, these different types of experiments on functionally eliminated livers or on experimental liver injuries point in the same direction as regards the influence on the C/s. It is evident, however, that the retardation of the Ci-breakdown in the kidneys is greater when an abundant absorption from the intestine is proceeding while the liver is out of function, or, if the liver is injured, while it is in communication with the circulation and toxic products can flow out. The Ci-elimination curves show a perfect parallel to what I found when urethane was given for narcosis intravenously instead of subcutaneously (MÅRTENSSON, 1938). Then the substance evidently accumulates in the renal parenchyma to so high a concentration that it directly inhibits the Ci-dehydrogenase through its narcotic effect.

Half an hour after intravenous injection of 25 % urethane to an amount of 1.5 gm per kg in a rabbit, the C/s was 62.8 γ in the arterial blood and 58.5 γ in the renal-vein blood. The difference amounts to only 7 % of the arterial value, which shows that the renal oxidation of Ci is greatly reduced.

Is the Ci-metabolism in vivo connected with the amino-acid metabolism?

As was previously mentioned, I have been able to show that administration of malic acid inhibits the breakdown of Ci in the kidneys (MÅRTENSSON, 1939). Possibly the same mechanism is behind the effect of all those substances which, on being added, have proved able to increase the Ci-excretion in the urine. Whether any of these metabolites contribute to elevating the C/s in injury of the hepatic cells is an open question until we have a better knowledge of their metabolic conditions in the body.

Another reaction suggests itself more readily if the cause of the C/s rise in hepatic lesion is sought in a specific inhibition, viz. glutamic-acid formation from Ci via α -ketoglutaric acid,

which according to ADLER and others (1939) is the most effective route for formation of amino-acids in the body. The breakdown of amino-acids seems to take place chiefly in the liver, and the latter's capacity to do this is reduced when this organ is injured. After intravenous injection of amino-acids (7 mgm amino-acid nitrogen per kg) to rabbits, the amino-

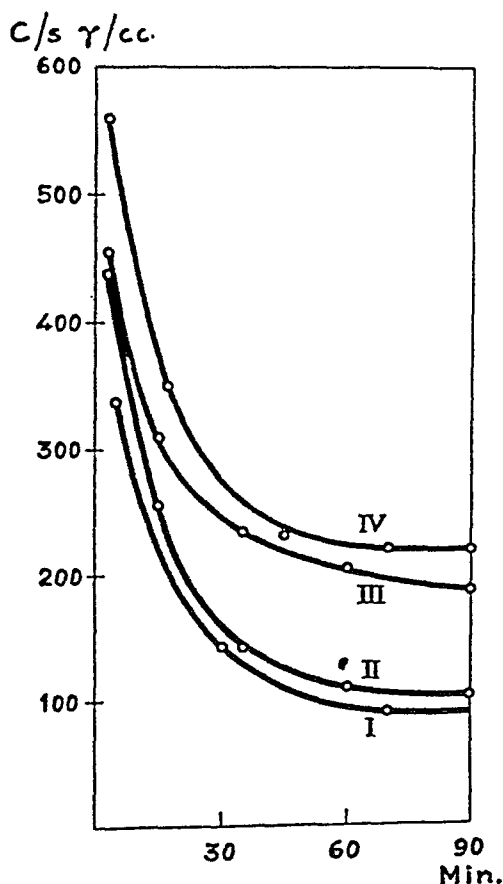


Fig. 15. C/s after intravenous administration of Ci.

- I. Rabbit, 2.1 kg. No operation. 60 mg Ci/kg.
- II. Rabbit, 4.0 kg. Ligation of arteries to portal area and of portal vein. 60 mg Ci/kg.
- III. Rabbit, 4.1 kg. Ligation of hepatic artery. 2 hours later 60 mg Ci/kg.
- IV. Rabbit, 3.0 kg. Same operation as II. 60 mg Ci and 100 mg glutamic acid/kg.

acid content of the blood already returns to the normal value after 30—60 minutes (KITAMURA, 1937), but if the liver is injured, especially by chloroform, the amino-acid content rises

and the tolerance curves assume an ascending character. The elimination, however, is as rapid if the kidneys are extirpated. This agrees with an investigation by SVEDBERG, MADDOCK and DRURY (1938): The amino-acid content remains normal after nephrectomy but rises vigorously after hepatectomy. In the course of necrotic processes in the liver, amino-acids are also formed during tissue disintegration, and as a result a considerable rise of the amino-acid level in the blood can be found in pronounced liver injuries. In such cases a likely assumption is that a displacement may occur of the equilibrium in the reversible reaction between Ci and glutamic acid with, as its result, a compensatory increase of the blood Ci in order to restore the reaction equilibrium.

I have therefore conducted some experiments to learn how addition of glutamic acid affects the *in-vivo* metabolism of Ci. ORTEN and SMITH (1937) had found only a slight increase of the Ci-concentration in the urine in dogs after intravenous injection of glutamic acid. But this slight effect is no doubt attributable to the very rapid breakdown of the amino-acids by the liver and their disappearance from the blood. In agreement with this, I found that the effect was slight after intravenous injection of glutamic acid (KAHLBAUM), 100 mgm per kg being administered to a rabbit (without narcosis).

12. 10. 39.

	Normal value	10	20	40 min. after inj.
C/s	71.4	75.2	79.6	81.8 γ /cc

After joint Ci and glutamic-acid administration the Ci-elimination was however found to be distinctly retarded, especially at first when the glutamic content of the blood could be assumed to be high.

16. 10. 39. Rabbit, 2.3 kg. No narcosis. Inj. of 100 mgm Ci per kg + 100 mgm glutamic acid per kg during exactly five minutes.

	Normal value	4	30	55	86 min. after inj.
C/s	88.7	525	352	207	169 γ /cc
	(87.6	510	230	150	135 „)

The bracketed figures are corresponding values from a control test with the same amount of Ci but without glutamic acid, likewise on a non-narcotized rabbit.

In order to escape the rapid break-down of glutamic acid, and to gain time for its effect to be exercised, I carried out the same experiment on a rabbit whose liver and portal area

had been functionally cut off (after this operation the amino-acid nitrogen in the blood rises by 1—2 mgm per 100 cc in two hours). After injection of 100 mgm of glutamic acid per kg (Sodium glutamate, mono, B. D. H.) the amino-acid nitrogen in the blood rose from 6.3 to 11.1 mgm per 100 cc and after a further 95 minutes was 12.3 mgm per 100 cc. The *Ci*-elimination curve after injection of 60 mgm of *Ci* per kg is given in Fig 15, curve IV, which should be compared with curve II as representing a control test without glutamic acid. As will be seen, the retardation is considerable. Direct measurement of the difference between the *C/s* of arterial and that of renal-vein blood gave a value of 17.5 %, which implies a considerable inhibition.

The amino-acid estimation was carried out by Folin's colorimetric method (FOLIN, 1922) with unlaked blood as starting-point according to FOLIN (1930). As standard I used a solution of glycine (Amino-acetic acid Analar, B. D. H.), and the amino-acid reagent was likewise from the B. D. H.

The amino-acid estimation in the investigations referred to above was made by the same method, and hence the values are comparable. The average value of the amino-acid nitrogen for the rabbit is 7.9 mgm per 100 cc according to KITAMURA (1937), 8.1 mgm per 100 cc according to SVEDBERG, MADDOCK and DRURY (1938).

Lastly, an experiment is submitted in which glutamic acid was added during perfusion of isolated cat kidneys.

16.11.39. Cat, 3.2 kg, wt. of kidney 31 gm. Perfusion fluid 220 cc heparinated blood with almost 20 % Tyrode's sol. Haematocrit reading 34 %. Min. vol. between 48 and 58 cc. Pressure increasing from 80—110 mm Hg. Urine secretion very slight during whole experiment. After a period for stabilization, *Ci* was added in continuous drip, 40 mgm during 20 min. Glutamic acid (neutralized), altogether 500 mgm, was then added, whereupon another 40 mgm of *Ci* was supplied during 20 min. The calculation of the *Ci*-consumption was effected as in previous perfusion tests. Blood vol. computed during both periods at 215 cc, the *Ci*-content of the perfusion blood in relation to the plasma in the first period at 75 %, in the second at 80 %, as a certain volume of fluid was added.

	<i>C/s</i>	Estimated <i>Ci</i> -consumption
1st period	25.7—160 γ /cc	18.4 mgm
2nd „	64.4—224 „	12.5 „

The breakdown of *Ci* in the kidneys thus fell, after addition of glutamic acid, by about 30 %, although the rise of the average *C/s* in the later period, from 93 to 144 γ /cc, ought to have increased the oxidation by 50 %. Judging from these

experiments there thus exists a *rather high probability that the reaction between Ci and glutamic acid can also play a rôle in vivo*.

Even if the amino-acids are not broken down in these later experiments, the possibility must be conceived of the glutamic acid being changed by the transamination that is constantly taking place (SCHOENHEIMER, RATNER and RITTENBERG, 1939). In the course of this, it is thought that alanine or aspartic acid is first formed (COHEN, 1939) and the glutamic acid is converted into α -ketoglutaric acid, which as a breakdown product of Ci ought to inhibit the further disintegration of this acid. Measurement of the amino-acid nitrogen in the blood should give a total picture of what happens even if a transamination takes place. However, I have not yet had an opportunity of investigating whether an amino-acid formation actually occurs during kidney perfusion with addition of Ci in such amount that it is able to give a clear verdict. In a preliminary experiment with anastomosis between the renal and jugular veins, the amino-acid concentration was certainly higher in the renal-vein blood after addition of Ci (8.4 mgm per 100 cc as against 7.9 mgm per 100 cc in the arterial blood), but the point was not closely investigated.

The following account shows how the C/s and the amino-acid content of the blood are changed after the different operations dealt with in this chapter. The related estimations of the C/s and the amino-acid nitrogen were made on the same blood samples.

1) Functional cutting-off of liver and portal area:

Rabbit: C/s	71.9—27.3—28.7	γ /cc
Amino N	...	7.4 — 8.5	mg p. 100 cc
Cat: C/s	31.0 — 42.2	γ /cc
Amino N	...	5.9 — 8.7	mg p. 100 cc

2) Functional cutting-off of liver but intact portal circulation:

Rabbit: C/s	61.7 — 107	γ /cc
Amino N	...	6.8 — 14.3	mg p. 100 cc
Cat: C/s	46.4 — 64.3	γ /cc
Amino N	...	5.4 — 7.2	mg p. 100 cc

3) Ligation of hepatic artery:

Rabbit: C/s	60.7 — 115	γ /cc
Amino N	...	7.3 — 11.3	mg p. 100 cc
Rabbit: C/s	59.6 — 141	γ /cc
Amino N	...	5.3 — 10.8	mg p. 100 cc

A definite parallel is thus noticeable between the degree of the rise in the amino-acid content and of that in the C/s, and hence it is possible that there is also a causal relation between them. In this way these experimental observations might lead

to the following view as to the reason for the applicability of the C/s determination as a liver-function test: The breakdown of Ci takes place chiefly in the renal parenchyma. The Ci-metabolism is, however, intimately associated with processes within the carbohydrate and protein metabolism, especially the transformation of amino-acids, which processes have the closest connexion with the metabolic function of the liver. Since Ci is a most readily convertible substance and occurs in very low concentration, it responds very sensitively to variations in the quantitative conditions of the reactions in which it takes part. For this reason we can employ the C/s as a sensitive indicator to the functional state of the liver, as we possess means of exactly registering even minor variations in the C/s.

Summary.

Estimation of the serum citric acid has proved a valuable aid for the differential diagnosis of hepatitis and obstructive jaundice. The hypercitricaemia attending injuries of the hepatic parenchyma cannot, however, according to experiments submitted in Chapter II, be due to a diminished Ci-oxidation in the liver, as was previously assumed.

Animal experiments show that if the liver is put out of function, or is injured, there occurs an inhibition of the Ci-oxidation in the kidneys, with a diminution of the difference between the C/s of the arterial blood and that of the renal-vein blood and a markedly retarded elimination of the added Ci.

Administration of glutamic acid retards the Ci-elimination and diminishes the Ci-oxidation in the kidneys in perfusion experiments. In those experiments in which the liver is put out of function, or is injured, there is a definite parallel between the rise in the C/s and that in the amino-acid content of the blood. Therefore, as a possible specific cause of the hypercitricaemia in injuries of the hepatic parenchyma, it is suggested that a deterioration of the amino-acid breakdown in the liver may bring about a displacement of the equilibrium in the reaction between the Ci-breakdown and the glutamic-acid formation, causing a rise in the C/s. Ci, being a substance that is very readily oxidized and that occurs in low concentration, responds very sensitively to every change in the reaction environment.

CHAPTER IV.

Some Experiments on the Ci-metabolism attending Changes in the Acid-base Equilibrium.

Earlier in this work it was mentioned on a couple of occasions that changes in the acid-base equilibrium may bring about alterations in the C/s and affect the experimental results. For this reason I am submitting below a short review of the literature on this question as well as some animal experiments, although I have not yet been able to make a detailed experimental inquiry into the problems involved.

ÖSTBERG's investigation (1931) shows that the Ci-excretion in man is always diminished after administration of acids or of agents producing acidosis as well as after carbohydrate starvation, while it is always increased after administration of alkalis. ÖSTBERG points out that this Ci-excretion in the presence of excess of alkalis acts sparingly on the "fixed" anions and can therefore play a part in the renal regulation of the acid-base conditions. At that time no definite opinion could be advanced as to the source of this Ci or as to the cause of the variations induced by changes in the acid-base equilibrium. A later investigation led ÖSTBERG (1934) to the conclusion that the Ci-excretory changes induced by administration of acids or alkalis cannot be governed by changes in the C/s, since no sure correlation could be established between them.

KUYPER and MATTILL (1933) confirmed ÖSTBERG's results. They were able to show that, in the rabbit, inanition causes the C/s to fall but that simultaneous administration of bicarbonate counteracts this fall. If ammonium chloride is supplied, the C/s drops considerably, though the authors ascribe most of the effect to the inanition. Alkalosis through hyperpnoea increases the Ci-excretion, powerful bodily movement diminishes it. The authors state that this diminution is evidently not a consequence of a decline in the C/s and that "there is as yet no information as to what the nature of the kidney threshold for citrate may be".

ÖSTBERG's results were further confirmed by BOOTHBY and ADAMS

(1934) who point out, however, that on the basis of the clinical material they have been unable to discover any correlation that would be of clinical significance.

LENNER (1934) reported a couple of experiments on man, according to which there is possibly a rise of the C/s after oral administration of bicarbonate, a fall after ammonium chloride.

SHERMAN, MENDEL and SMITH (1936 a) likewise confirm that the urinary Ci varies in amount direct with the pH of the urine, irrespective of the cause of the pH variations. The increase in the Ci-excretion following intake of bicarbonate is especially marked (up to 100 times) in dogs and rats with their normally very low Ci-concentration in the urine. The authors infer that the increased Ci-excretion is not caused by an increase of the C/s but must be due to a specific activity of the kidneys.

This remarkable phenomenon in Ci-excretion associated with change in the acid-base equilibrium, and the specific renal activity underlying it, can now be readily explained, since my experiments have shown that Ci is being constantly supplied in relatively large quantities to the kidneys and there oxidized, while in normal cases only a small proportion of it is excreted. The Ci which after administration of alkali is excreted in increased amount is thus readily available on the spot. A rearrangement of the renal activity in the service of the acid-base regulation may involve a change in the relative proportion of Ci-breakdown and Ci-excretion without necessarily altering the total elimination of Ci in the kidneys.

Some experiments on rabbits, however, showed that *an acidosis produced by ammonium chloride or calcium chloride can depress the C/s very considerably.*

14. 11. 39. Rabbit, 4 kg. Administration through stomach-sound of 12 cc of 25 % ammonium chloride = 0.75 gm/kg.

	Normal value	3 1/2	6 1/2	22	31	55 hrs after admin.
C/s	68.2	52.2	41	26.5	28.5	47.9 γ/cc

In this case inanition cannot play any rôle, for the rabbit had already starved 24 hours before the beginning of the experiment and was given food again 22 hours after the ammonium chloride was supplied. In spite of this the C/s kept low for a long time. (The pH of the urine, tested with Lyphan paper, dropped to 5.3).

30. 11. 39. Rabbit, 4 kg. Administration through stomach-sound of 30 cc of 10 % ammonium chloride = 0.75 gm/kg.

	Normal value	5 1/2	16 1/2	20 hrs after admin.
C/s	90.1	44.9	27.9	26 γ/cc

The urinary Ci-concentration fell from 167 γ /cc (pH 8.5) to 31.8 γ /cc (pH 5.3).

11. 12. 39. Rabbit, 4 kg. Administration through stomach-sound of 40 cc of 10 % $\text{CaCl}_2 = 1 \text{ gm/kg}$.

	Normal value	22	38	74	hrs after admin.
C/s	33.7	19	25.1	51.1	γ /cc

The urinary Ci-concentration dropped from 71.9 γ /cc (pH 8.3) to 29 γ /cc (pH 5.2). On autopsy a few days later it was found that the rabbit had ascites and swollen lymph-glands in the abdomen, this being perhaps the cause of the low initial value.

After oral administration of sodium bicarbonate to a rabbit no distinct elevation of the C/s was observed.

21. 11. 39. Rabbit, 2.7 kg. Administration through stomach-sound of 20 cc of 10 % sodium bicarbonate = 0.75 gm/kg.

	Normal value	2 1/2	6	12	hrs after admin.
C/s	93.5	97.8	99.7	92.6	γ /cc

At the beginning of the experiment the urine was already so alkaline (pH 8.4) that no further rise of the pH ensued. The urinary Ci-concentration rose from 176 to 305 γ /cc.

On the other hand, the elimination of Ci from the blood was distinctly retarded when Ci was given at the same time as sodium bicarbonate was infused intravenously, although the Ci-excretion in the urine was considerably larger than normally. Simultaneous measurements of the C/s in the arterial and renal-vein blood showed a clear retardation of Ci-breakdown in the kidneys.

12. 5. 40. Rabbit, 5 kg. Urethane 1.25 gm/kg. Continuous intravenous injection of sodium bicarbonate, 0.35 gm/kg per hour. After administration of 100 mg Ci per kg the C/s was as follows:

	Normal value	3	15	35	60	90	120 min. after inj.
C/s	80.5	585	380	282	183	138	120 γ /cc

During the experiment 93 mgm of Ci, or 18.6 % of the added amount, passed away with the urine (Cf. experiment in Fig. 11). At the last sampling the C/s of the renal-vein blood was only 15.8 % lower than that of the arterial blood.

In a couple of preliminary experiments on perfused isolated cat-kidneys I have studied the changes that take place in the Ci-metabolism when the reaction in the perfusion fluid is displaced by changes in the carbon-dioxide content of the gas-

mixture bubbling through. This procedure is the only safe one if the intracellular hydrogen-ion-concentration is also to be affected.

AHLGREN (1930) demonstrated the importance it has for experiments on surviving organs to maintain the bicarbonate- CO_2 buffer system in the serum saline solution: if air or pure oxygen is passed through a serum saline solution containing bicarbonate, the pH is rapidly displaced to the alkaline side. A displacement of this kind also occurs if blood is used as an experimental medium, although it is there counteracted to some extent by the great buffering capacity of the blood. In an experiment conducted by HASSELBALCH the carbon-dioxide tension was doubled without change in the bicarbonate content, the pH of the total blood falling by 0.19 instead of the theoretical 0.30 value.

My experiments were arranged in the following way: After a stabilizing period, 30 mgm of Ci was added during 20 minutes, the "normal" gas-mixture, $\text{O}_2 + 5\% \text{CO}_2$, being meanwhile bubbled through the perfusion blood. Then the gas-mixture was altered to $\text{O}_2 + 10\% \text{CO}_2$, and after an adjustment-period of ten minutes another 30 mgm of Ci was added during 20 minutes. Thereafter the Ci-addition was repeated once more, now with a gas-mixture of $\text{O}_2 + 1\% \text{CO}_2$. The consumption of Ci during the different test-periods proved to be as follows:

$\text{O}_2 + 5\% \text{CO}_2$	$\text{O}_2 + 10\% \text{CO}_2$	$\text{O}_2 + 1\% \text{CO}_2$
17.2 mg	22.6 mg	19.5 mg Ci

In another experiment this change of the buffer system was inversed. In each test-period here 40 mgm of Ci were added. The Ci-consumption during the different periods was:

$\text{O}_2 + 5\% \text{CO}_2$	$\text{O}_2 + 1\% \text{CO}_2$	$\text{O}_2 + 10\% \text{CO}_2$
29.7 mg	25.7 mg	29.4 mg Ci

According to these experiments the Ci-consumption in the kidneys is increased by a displacement of the reaction in an acid direction and diminished by a displacement in the alkaline direction. The changes are not especially great, but may be considered as certain. And a relatively small change in the Ci-breakdown can in course of time alter the C/s very considerably. For that quantity of Ci which is excreted in the urine a small change of the oxidation may play a great rôle, as normally this part is a very small proportion of the total conversion. According to the conception of the course of events, to which these experiments give rise, the changes in the C/s and the Ci-excretion are not directly dependent on each other but both are determined by the intensity of the Ci-oxidation in the renal parenchyma.

In this field of inquiry, however, there remain many questions unsolved. It is highly probable that the Ci-metabolism is bound up with the amino-acid metabolism. The question may then be whether an increased Ci-oxidation depends upon a supply of H_2N , such as when ammonium chloride is administered or when the ammoniac defences of the kidneys are brought into play in acidosis. ÖSTBERG's (1931) curves show with all clearness that the amount of Ci in the urine is inversely proportional to that of the ammoniac. KREBS and COHEN (1939) state that addition of H_4NCl to renal cortex in *in-vitro* experiments markedly increases glutamic-acid formation and respiration in presence of α -ketoglutaric acid or of substances that can form this acid. I have found no distinct increase of the Ci-elimination after administration of H_4NCl direct intravenously in rabbits, but the strong central irritation associated with such probably disturbs the experimental conditions, and this question should therefore preferably be studied by perfusion tests on kidneys.

I have not closely studied the question of whether an acidosis also brings about a diminished Ci-formation in the body. In an experiment in which acidosis was induced with ammonium chloride, the C/s was 30.1 γ per cc in the arterial blood, 17.6 γ in the renal-vein blood. This difference certainly implies a powerful Ci-oxidation in the kidneys, but absolutely it is not so large that a Ci-formation equal to the normal can be expected.

Nor has it been elucidated whether the Ci-metabolism in those pathologic conditions in which the C/s is generally low (postoperative conditions, thrombosis and inflammations) is associated with an acidosis, as has been surmised, or with changes in the protein metabolism. The postoperative drop in the C/s must, in any case, also depend on something other than an increased renal oxidation of Ci, since in the rabbit it also occurs after bilateral nephrectomy (See Fig. 14). In two rabbits whose kidneys had been extirpated, the C/s fell considerably towards the end, when the animals exhibited signs of severe inflammation of the intestine.

The vigorous decline of the C/s after administration of salicylic acid (ALWALL, 1938) does not appear, according to later investigations, to be due to a direct salicylic action but to an acidosis produced by salicylic acid (ALWALL and MÅRTENSSON, unpublished).

Summary.

Earlier research-workers have shown that administration of acids or of agents producing acidosis diminishes the Ci-excretion, while administration of alkali increases it. These changes in the Ci-excretion could not be explained by corresponding falls or rises of the C/s, but were assumed to be due to a specific renal activity. The situation is now amenable to ready explanation, since my experiments have shown that

the Ci normally excreted is merely a small proportion of that oxidized in the renal tissue. Under the influence of a rearrangement of the renal activity in the service of the acid-base regulation, a displacement of the proportion between Ci-breakdown and Ci-excretion can therefore occur without necessarily affecting the C/s.

An acidosis brought on by ammonium chloride or calcium chloride can also depress the C/s very materially in the rabbit. Administration of bicarbonate gives a doubtful rise of the C/s, but retards the Ci-elimination, the Ci-excretion being then increased. The Ci-metabolism in the renal parenchyma, studied by means of perfusion tests on isolated cat-kidneys, rises in response to displacement of the reaction in an acid direction, falls in response to displacement in an alkaline direction. It is therefore possible that changes of the Ci-concentration in both blood and urine are secondary phenomena to a change of the Ci-oxidation in the renal parenchyma.

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